Physiological Zoölogy

Vol. XV JULY 1942 *No. 3*

A QUANTITATIVE STUDY OF THE EFFECTS OF DIFFERENT CONCENTRATIONS OF ETHYNYL TESTOSTERONE AND METHYL TESTOSTERONE IN THE PRODUCTION OF GONOPODIA IN FEMALES OF GAMBUSIA AFFINIS[1]

(Fourteen figures)

C. L. TURNER
Department of Zoölogy, Northwestern University

THE gonopodium of the males of poeciliid fishes is a modified anal fin used in copulation. Before the onset of sexual maturity, the fin, almost identical in structure in males and females, is a generalized organ with ten bony rays. The approach of sexual maturity in the males of *Gambusia affinis* is marked by a rapid metamorphosis of the fin, in which there is a characteristic acceleration of growth in rays 3, 4, and 5 and a subordination of growth in the other rays (Turner, 1941*b*). During the elongation and segmentation of rays 3, 4, and 5, two differentiation areas arise, the first (Area I) having the effect of thickening the third ray, and the second (Area VI) producing a fusion and a thickening of the bifurcated portions of rays 6, 7, and 8 and the portion of ray 5 at the same level. Near the close of the period of elongation of the 3, 4, 5-ray complex, four other differentiation areas appear in a definite sequence. Area II produces a series of posteriorly directed spines and plates on the terminal fifteen segments of ray 3. Area IV involves the terminal segments of the dorsal branch of ray 4 and the ventral branch of ray 5. Terminal hooks appear on each of these members and become closely associated. Beginning about four segments from the end of the dorsal branch of ray 4 and extending forward for approximately seven segments, a series of curved, vertical serrae are formed on the segments. This series of serrae constitutes Area V. Area III, the "elbow," takes the form of a series of low spines or a blade arising from the second to the fourth segments of the ventral branch of ray 4 a few segments from the end. Areas I and VI appear and continue their development during the growth and segmentation of the 3, 4, 5-ray complex. Areas V, IV, and III appear, in the order named, at the conclusion of the growth period. The details of the differentiated structures and the arrangement of the areas in the mature gonopodium are illustrated in Figure 9.

[1] This investigation has been supported in part by a grant-in-aid from the Graduate School of Northwestern University.

The structural pattern of the bony elements in the gonopodium is characteristic for each species of poeciliid fish, and in each species it may be assumed that there is a group of distinct genetic factors for the characteristic structures. In all species, however, metamorphosis of the anal fin is characterized, first, by rapid growth and segmentation of three or four rays, during which there may be some thickening of the rays and some fusion of their branches, and, second, by a period of further differentiation during which the characteristic spines, plates, hooks, serrae, etc., arise. With the onset of the late period of differentiation, growth of all rays is terminalized, and at the conclusion of the period of differentiation there is no further development of any kind in the gonopodium. Body growth in the male fish is terminalized also; and the mature male is usually small in contrast to the mature female, in which body growth and growth of the anal fin continue throughout life. It follows that all species of the poeciliid fishes possess a common factor for, first, stimulation of growth and segmentation in the 3, 4, 5-ray complex and, second, the origin of the differentiation areas in the fin field, within which arise the characteristic gonopodial characters of the species. It would appear evident also that an agent which would produce growth and differentiation of the fin in one species would produce the same result in other species. Experimentation upon a number of species with several androgenic hormones has demonstrated that this conclusion is correct.

Various investigations now indicate that a factor immediately responsible for sex dimorphism in the anal fin is an androgenic hormone released from the testis during its development and, further, that genetic factors for the specific characteristics of the gonopodium exist in females as well as in males but that, lacking the androgenic hormone, the females fail to develop the specific characters. Regnier (1937) was able to reverse the sex in females of *Xiphophorus helleri* by injection of testosterone propionate, and with the reversal of sex the anal fin assumed the male form in part. Grobstein (1940) obtained similar results with testosterone propionate in *Platypoecilus maculatus* and in addition showed that a more complete resemblance to the male fin is produced if the female fin is cut back and allowed to regenerate while the female is treated with the hormone. Baldwin and Goldin (1939) treated females of *X. helleri* with testosterone propionate and secured a modification of the gonad, as well as the development of male secondary sex characters. Eversole (1939, 1941) was able to cause the production of atypical gonopodia in females of *Lebistes reticulatus* at various ages by treating them with ethynyl testosterone and testosterone propionate. He demonstrated also that the time required for gonopodial development was shorter when 5–10 mg. of hormone was added to a tank each week, in contrast to the effects secured by adding only an initial amount of 5 mg. Turner (1941*c*), in a series of experiments in which portions of fins and whole fins were excised and allowed to regenerate after gonopodial metamorphosis had started, demonstrated that the pattern of the regenerating gonopodium was changed to some extent because of a testicular hormone released during the regeneration. In *G. affinis* females it has been shown also (Turner, 1941*a*) that the extent to which the anal fin may be made to transform into a gonopodium by treatment of a female with ethynyl testosterone in high dosages depends upon the extent to which the fin has already been fixed in the female pattern and the extent to which the induced growth has taken place before differentiation occurs. St. Amant (1941) demonstrates conclusively that complete castration of males before metamorphosis of the gonopodium prevents metamorphosis and that complete castration during metamorphosis prevents further development of the gonopodium. Treatment of the castrated animals with ethynyl testosterone causes them

to renew and to complete gonopodial development. He shows, further, that very small amounts of testis are capable of stimulating the development of early stages of metamorphosis of the gonopodium, that larger quantities are necessary for development of some of the differentiation areas, and that still larger amounts are necessary for the completion of development. A piece of testis about 1/45 of the normal volume is capable of producing enough hormone for complete development. However, the time required in such a case is considerably extended.

The results of St. Amant's work, together with those secured by treating females with high dosages of ethynyl testosterone, suggest not only that in the development of normal males the testis furnishes an androgenic hormone necessary for the metamorphosis of the fin but also that the small amount necessary for the initial stages must be augmented for the production of complete metamorphosis. The failure of the testis to produce more than the initial amount would result in cessation of metamorphosis. This hypothesis could be tested by castrating males before the beginning of metamorphosis of the fin and subjecting the castrated males to a varied series of concentrations of an androgenic hormone. Since the anal fins of females readily undergo a metamorphosis when the females are treated with ethynyl testosterone and since during the metamorphosis the responses in growth and formation of specific differentiation areas are the same as those in the male, it was decided to study, first, the effects of ethynyl testosterone and methyl testosterone[2] in different concentrations upon the metamorphosis of the fin in the female. Presumably it would be possible to determine the concentration of the hormone necessary to promote development and also the level of hormone which would be adequate for complete metamorphosis. The results would be evident in terms of the time required for the development of the stages and in relative completeness of development.

The results cannot be interpreted as exactly representing the hormone levels required for development of the fin of the normal male. The ovary may be secreting an antagonistic hormone as indicated by the work of Berkowitz (1938) and Regnier (1938). A more critical series of results would be obtained if the females were ovariectomized before they were treated with ethynyl testosterone.

Experimentation has shown that time is a reliable criterion for indicating stages of development only if temperature is controlled. It has been demonstrated also that the reactivity of specimens varies with age. Consequently, experiments were conducted under conditions of uniform temperature (25°–27° C.) upon specimens within a narrow age range. All specimens were between 23 mm. and 27 mm. in standard length.

The hormone concentrations were made up by adding water containing the hormone to the water of the various aquaria so that the total volume of water in each aquarium contained a known concentration of hormone. In earlier experiments (Turner, 1941*a*) it was found that a concentration composed of 1 mg. of hormone to 1,500 cc. of aquarium water brought about a very precocious metamorphosis of the anal fin and produced structural abnormalities. It was assumed that such a concentration was much too high for normal development. Consequently, a range of concentrations was used varying from 1 mg. of hormone in 2,000 cc. of water to 1 mg. in 20,000,000 cc. of water. It was learned also that hormone concentrations in the aquaria became reduced during the progress of the experiments. In order to keep the hormone at an approximately constant level the water in each aquarium was changed every two days, and a fresh solution of

[2] Supplies of ethynyl testosterone and methyl testosterone were generously donated by the Schering Corp. of Bloomfield, N.J.

hormone of the desired concentration was added. The process by which the fish take up the hormone from the water is not known exactly. Eversole (1941) suggests that ethynyl testosterone is taken into the alimentary canal during feeding. The hormone is known to be orally effective in mammals. The hormones are slightly soluble in water,[3] however, and there is a possibility that they may be absorbed through membranes other than those of the alimentary tract.

Specimens were examined at the beginning of the experiments, and the ray-segment formulas (Turner, 1941*b*) were tabulated. Daily examination of the specimens was made for evidence of growth and differentiation.[4]

NORMAL DEVELOPMENTAL STAGES

Arbitrary stages which could be used in following the development of the gonopodia specimens were selected.

Stage 1.—The first reaction consists of a change in vascularity in the soft part of the fin, particularly along the ventral surface of the third ray and the region near the ends of rays 4 and 5. The capillary loops increase in diameter in localized portions, so that a series of swellings is produced. The circulation in these portions becomes sluggish and finally stagnates completely. The stagnated blood appears a little later as a group of isolated islands, large and small, which are cut off temporarily from the vascular loops in which circulation is active. At the same time there is a local tissue proliferation, which results in a considerable thickening of the soft parts in these areas. A milky opacity is evident when the tissue is examined macroscopically.

Stage 2.—A rapid elongation of rays 3, 4, and 5, accompanied by segmentation of the rays, follows the first reaction. At the same time in differentiation Areas I and VI there is a beginning of a modification of portions of the juvenile fin, which have been formed previously. Within Area I there is a thickening of the segments of ray 3, and within Area VI there is a fusion of the branched portions of rays 6, 7, and 8 and of ray 5 at the same level.

Stage 3.—In the development of the normal male, fin growth and segmentation in ray 3 is gradually terminated with the appearance of differentiation Area II. The appearance of this area is marked by a progressive decrease in the thickness of the ray in the last fifteen segments and an addition to each segment ventrally of a plate or spine (Fig. 9). The beginning of the development of the structures in this area is used as stage 3.

Stage 4.—The first appearance of the series of vertical serrae in differentiation Area V upon the dorsal branch of ray 4 represents stage 4 (Fig. 9).

Stage 5.—Within Area IV terminal hooks develop on the dorsal branch of ray 4 and the ventral ramus of ray 5 (Fig. 9). In some of the treated females there is a failure of the development of the hook on ray 5, and in most specimens the hook develops later than the one on ray 4 (Fig. 9). However, the first appearance of the hook on ray 4 or ray 5 is used to represent stage 5.

Stage 6.—The elbow developing in Area III on the ventral branch of ray 4 (Fig. 9) is the last of the structural characters to appear in normal males. In some males it does not develop at all. Stage 6 is represented by the first appearance of this element.

[3] Dr. Byron Reigel determined the solubilities. One hundred cc. of water will dissolve 4 mg. of methyl testosterone or 0.7 mg. of ethynyl testosterone.

[4] The writer is indebted to Miss Ruth Irwin and Miss Peggy Uddbom for valuable technical assistance.

The duration of time required for the complete development of the structures indicated above varies; but this fact is not significant for the establishment of arbitrary stages, since only the first appearance of a structure is used to indicate the stage.

RESULTS OF EXPERIMENTS

REACTIONS OF MALES AND FEMALES OF THE SAME AGE

At effective temperature levels males at any age react more quickly than females of the same age. The testes in males which are already undergoing fin metamorphosis are producing hormone sufficient for the development of that stage; and the addition of a very low concentration of ethynyl testosterone brings the total amount of hormone to a higher level, with the result that the later stages of metamorphosis requiring this level develop prematurely. If high concentrations of hormone are added to the water, the time required for completion of development is much abbreviated, and structural abnormalities appear because of the early development of the differentiation areas in a fin which has not completed its growth.

In very young males (10–12 mm. in length) treated with a low concentration of hormone there is an accelerated development of the testis. The earlier development of the testis is responsible presumably for an early output of hormone. This early output from the testis, together with the constant additional supply of hormone in the water, combines to produce an amount in excess of that available to a treated female of the same age, with the result that gonopodial development in males of this age is much more rapid than in females. It has been found also that males at this juvenile stage are sensitive to a lower concentration of hormone than are females.

FEMALES TREATED WITH ETHYNYL TESTOSTERONE AND METHYL TESTOSTERONE

a) *Time of appearance of stages at different hormone concentrations.*—The results in part of treating medium-sized females with ethynyl testosterone and methyl testosterone will be seen in Figures 1 and 2. Attention is directed at this point particularly to the time in which each stage appears. The duration of the development of each stage and the causes for the abnormalities in the structures will be discussed later. Under ethynyl testosterone treatment at a concentration of 1 mg. of hormone to 20,000 cc. of water, development of all stages is abnormal to some extent, but all stages arise in the same sequence as in normal males. Decreasing the hormone concentration has little effect in the time of the appearance of the stages up to a concentration of 1 mg. to 500,000 cc. of water. At a concentration of 1 mg. in 1,500,000 cc. of water the interval between the first appearance of additional segments (stage 2) and the origin of the differentiation Area II (stage 3) is materially lengthened, and the other stages appear later than at higher concentrations. At a concentration of 1 mg. in 2,000,000 cc. of water the time interval between the appearance of stages 2 and 3 is still greater, and the appearance of the other differentiation areas represented by stages 4, 5, and 6 is postponed still further. Stages 5 and 6 fail to appear at all at a concentration of 1 mg. in 3,000,000 cc. Specimens were maintained under experimental conditions for sixty days, but there was no further development after twenty-seven days. At a concentration of 1 mg. in 5,000,000 cc., stage 3 appears in approximately twenty days, but stages 4, 5, and 6 do not appear at all. Stage 3 fails to appear at a concentration of 1 mg. in 10,000,000 cc., and at a concentration of 1 mg. in 15,000,000 cc. there is no induced development of any kind. If hormone

treatment is discontinued after partial development has been secured, development continues for several days and then ceases.

The results indicate the following facts: (1) Under hormone treatment the stages in the anal-fin development of the female occur in the same sequence as in gonopodial development in the normal male. (2) A specific concentration of hormone is required for the induction of each stage. (3) Those stages which appear last in the development of the gonopodium in a normal male require the greater concentrations of hormone in the development of the anal fin of the female, and those which appear first in the normal male

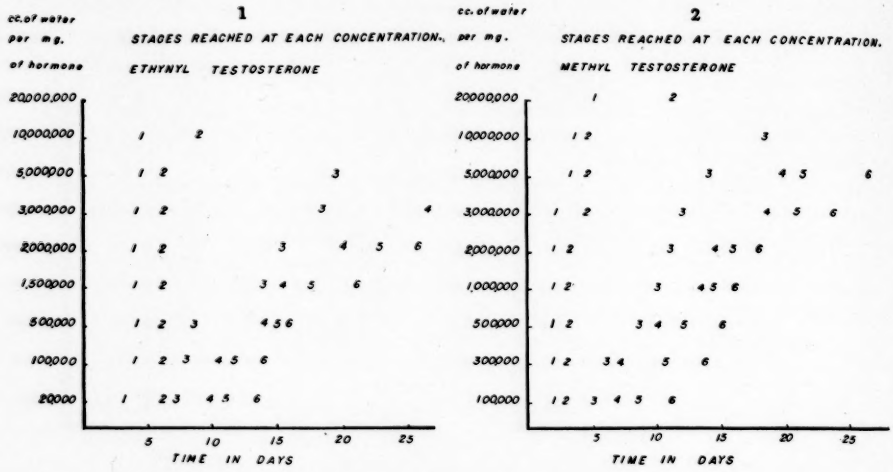
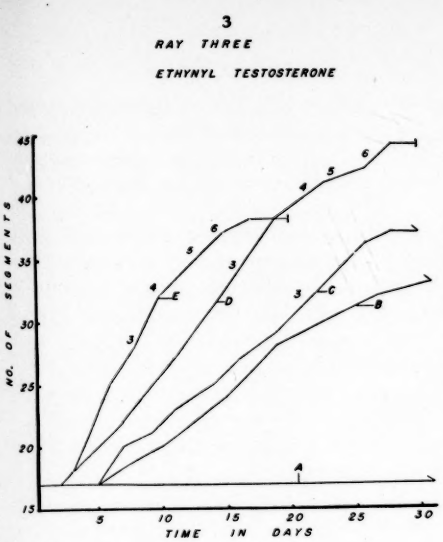


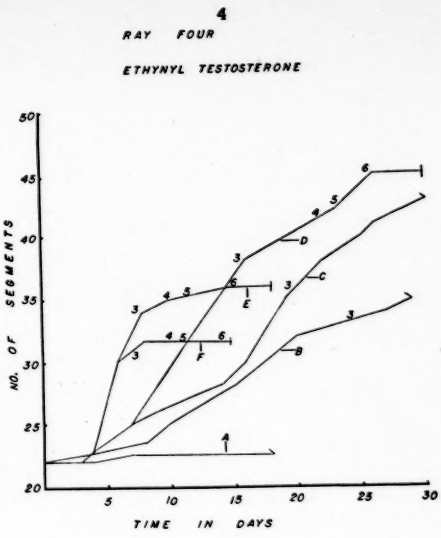
FIG. 1.—Time of appearance of each developmental stage in the metamorphosis of a medium-sized female of *G. affinis* treated with different concentrations of ethynyl testosterone. Numbers indicate the stages.

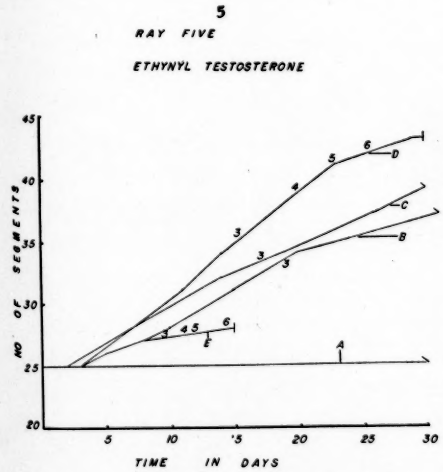
FIG. 2.—Time of appearance of each developmental stage in the metamorphosis of a medium-sized female of *G. affinis* treated with methyl testosterone at different concentrations. Numbers indicate the stages.

require the least concentration. (4) Unless a sufficient concentration of hormone is present, the various stages do not appear at all; and with progressive decrease in hormone concentration there is a progressive failure of the later stages to develop. (5) More time is required progressively for the induction of a given stage at concentrations progressively less than 1 mg. in 500,000 cc. of water. (6) A concentration greater than 1 mg. in 15,000,000 cc. is required for any reaction in the anal fin of the female.

If it be assumed that the hormone requirements for gonopodial development in the male are parallel to those for the same sequence of developmental stages in the induced anal-fin development in the female, it would follow that in the male a minute quantity of androgenic hormone is secreted by the testis at an early stage and that the amount is sufficient to initiate the early phases of fin metamorphosis, i.e., the vascular changes (stage 1) and the beginning of the elongation and segmentation in rays 3, 4, and 5 (stage 2). A greater output would be required for the development of stage 3 and a still higher







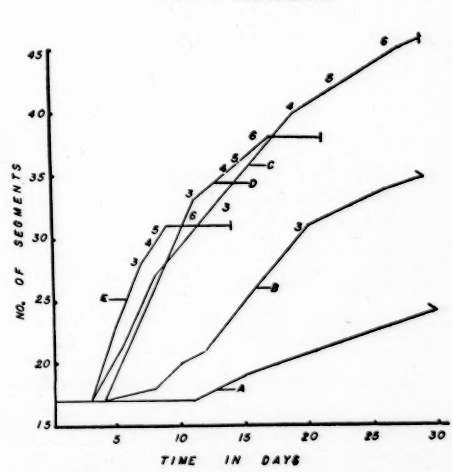


FIG. 3.—Number of segments added in ray 3 and time of appearance of developmental stages in female *G. affinis* treated with different concentrations of ethynyl testosterone. Stages are represented by numbers. Concentrations are as follows: $A = 1$ mg./20,000,000 cc.; $B = 1$ mg./10,000,000 cc.; $C = 1$ mg./5,000,000 cc.; $D = 1$ mg./2,000,000 cc.; $E = 1$ mg./100,000 cc.

FIG. 4.—Number of segments added in ray 4 and time of appearance of developmental stages in female treated with ethynyl testosterone. Concentrations *A*, *B*, *C*, *D*, and *E* are the same as in Figure 3. Concentration $F = 1$ mg./10,000 cc.

FIG. 5.—Number of segments added in ray 5 and time of appearance of stages in female treated with ethynyl testosterone. Concentrations *A*, *B*, *C*, *D*, and *E* are the same as in Figure 3.

FIG. 6.—Number of segments added in ray 3 and time of appearance of developmental stages in *G. affinis* treated with methyl testosterone. Stages are indicated by numbers. Concentrations: $A = 1$ mg./20,000,000 cc.; $B = 1$ mg./10,000,000 cc.; $C = 1$ mg./5,000,000 cc.; $D = 1$ mg./1,000,000 cc.; $E = 1$ mg./100,000 cc.

concentration of hormone for the development of stages 4, 5, and 6, successively. The rapidly developing testis would furnish the hormone in the concentrations required. If by any experimental procedure, such as castration, the source of the hormone should be removed, the gonopodium would cease to develop further but would not undergo regression.

When medium-sized females are treated with methyl testosterone, the anal fin is induced to develop the features of a gonopodium in the same manner as when specimens are treated with ethynyl testosterone. No differences in structure in general or in detail can be detected between the fins of females treated with the different hormones. How-

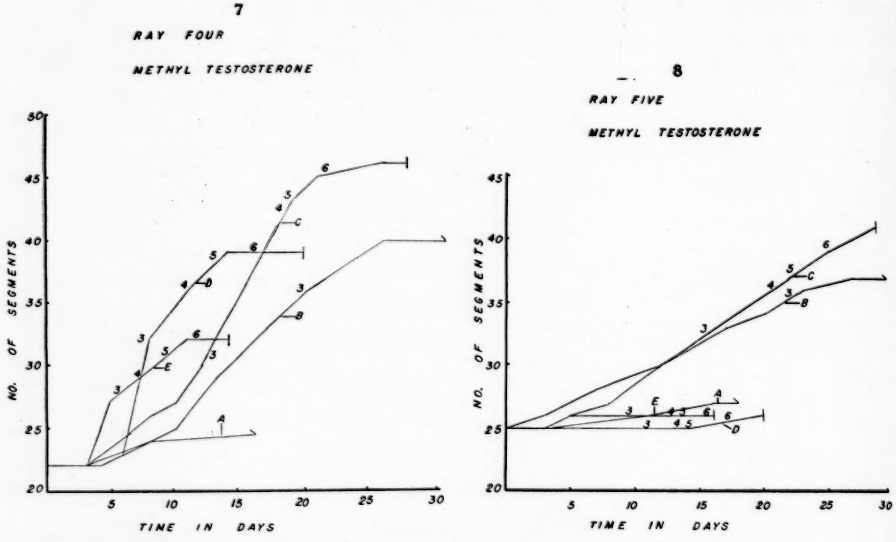


FIG. 7.—Number of segments added in ray 4 and time of appearance of stages in female treated with methyl testosterone. Concentrations *A*, *B*, *C*, *D*, *E* are the same as in Figure 6.

FIG. 8.—Number of segments added in ray 5 and time of appearance of developmental stages. Treatment of female with methyl testosterone. Concentrations *A*, *B*, *C*, *D*, *E* the same as in Figure 6.

ever, the concentrations required for the development of the different stages with methyl testosterone are much less than with ethynyl testosterone (Figs. 1 and 2). All stages appear with methyl testosterone at a dilution of 1 mg. in 5,000,000 cc. of water, while stages 4, 5, and 6 fail to appear at this concentration with ethynyl testosterone. Stages 1 and 2 appear in a concentration of 1 mg. in 20,000,000 cc. with methyl testosterone, but at a concentration of 1 mg. in 30,000,000 cc. there is no reaction of any kind. In this experimental material, therefore, it appears that methyl testosterone is between two and three times as potent as ethynyl testosterone in its capacity to induce the development of male characters in the anal fin of the female.

b) *Differential growth and segmentation in the rays of the 3, 4, 5-ray complex.*—In Figures 1 and 2, stage 2 marked the beginning of growth and segmentation in the 3, 4, 5-ray

complex. Duration of growth was not indicated. A study of the duration and extent of growth and segmentation brings out some interesting relations between hormone concentration and growth and between growth and differentiation. In Figures 3–8 the number of segments added to each ray of the 3, 4, 5-ray complex under the influence of different concentrations of hormone are shown, together with the time required for the development of each of the six developmental stages. In general the number of segments added may be taken as indicative of the degree of axial growth taking place. However, the two are not exactly parallel, for the reason that shorter segments are produced in higher hormone concentrations and also that the first few additional segments at any effective level of concentration are shorter than those which are produced later (Fig. 10). The production of short segments during early treatment and of longer segments later suggests that the effect of the hormone upon the fish is rather violent at first but that a physiological adjustment of some kind occurs in the animal shortly afterward.

Figure 3 shows graphically the number of segments added to ray 3 at different concentrations of ethynyl testosterone. The lines *A–E* represent concentrations as follows: *A* = 1 mg./20,000,000 cc.; *B* = 1 mg./10,000,000 cc.; *C* = 1 mg./5,000,000 cc.; *D* = 1 mg./2,000,000 cc.; *E* = 1 mg./100,000 cc. The numbers indicate the points at which each of the different developmental stages appeared. Arrows at the ends of the lines are used where complete development failed to occur, while short vertical lines at the ends indicate completion of growth and differentiation. All specimens used had reached the seventeen-segment stage before treatment was begun. An analysis of the results shows that (1) the higher the concentration of hormone, the shorter is the time required for the ray to acquire a given number of new segments and the later stages of differentiation; (2) a concentration of 1 mg./20,000,000 cc. is not effective; (3) a concentration of 1 mg./5,000,000 cc. stimulates the ray more than a concentration of 1 mg./10,000,000 cc., but neither concentration is effective in producing the terminal structural differentiations represented by stages 4, 5, and 6; (4) a concentration of 1 mg./2,000,000 cc. not only produces the largest number of new segments but is sufficient for the production of all stages of differentiation; (5) the number of segments added at a concentration of 1 mg./100,000 cc. is less than that at 1 mg./2,000,000; (6) at concentrations sufficient to produce all stages of differentiation the effect of the origin of the later differentiation stages is to terminalize the process of segment addition to the ray. It may be concluded that there is an optimum concentration for the growth and segmentation of the ray. A concentration of less than 1 mg. in 2,000,000 cc. is not sufficient to produce the maximal number of segments, and a concentration of more than 1 mg. in 2,000,000 cc. produces the development of the differentiation areas precociously, with the result that growth and segment addition are prematurely terminalized.

Growth and the addition of new segments in ray 4 follow the same general pattern as in ray 3, when the female is treated with the same concentrations of ethynyl testosterone. Ray 4 is branched, and the number of added segments indicated in the graph (Fig. 4) is an average of the segments added in both branches. In this figure, line *F* represents a concentration of 1 mg. in 10,000 cc., and the other lines represent the same concentrations as in Figure 3. It will be noted that ray 4 is slightly less susceptible to stimulation than ray 3, and at practically all concentrations the number of segments added is somewhat less. At a high concentration—1 mg. in 100,000 cc.—the terminalization of growth by the development of the differentiation areas is more pronounced than in ray 3.

In the ventral branch of ray 5 (Fig. 5) the response to stimulation as represented by

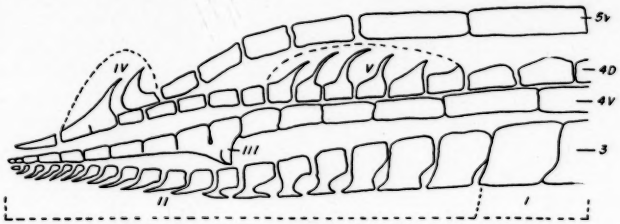
growth and segmentation is neither so rapid nor so extensive as in rays 3 and 4, but the terminalizing effect upon growth of high concentrations of hormone is very marked. There is no response at a concentration of 1 mg. in 20,000,000 cc. Increasing the concentration to 1 mg. in 10,000,000 cc. and 1 mg. in 5,000,000 cc. stimulates growth and segmentation, but the optimal concentration for maximal growth and for the production of the differentiation areas occurs at a concentration of 1 mg. in 2,000,000 cc. At a concentration of 1 mg. in 100,000 cc. only three segments are added. It is evident that the effect of the development of the differentiation areas is to terminate growth and segmentation quickly.

A comparison of the reactions in rays 3, 4, and 5 indicates that (1) the optimal concentration for all rays is approximately 1 mg. in 2,000,000 cc. of water; (2) the response in ray 5 is not so rapid as in rays 3 and 4; (3) the effect of retardation and terminalization of growth by the development of the differentiation areas at concentrations higher than optimal is greater in ray 4 than in ray 3 and much greater in ray 5 than in either ray 3 or ray 4.

The results obtained by the use of methyl testosterone are shown in Figures 6, 7, and 8. In each figure line *A* represents a concentration of 1 mg. in 20,000,000 cc., *B* of 1 mg. in 10,000,000 cc., *C* of 1 mg. in 5,000,000 cc., *D* of 1 mg. in 1,000,000 cc., and *E* of 1 mg. in 100,000 cc. The response of ray 3 is shown in Figure 6, of ray 4 in Figure 7, and of ray 5 in Figure 8. The responses are very similar to those obtained with ethynyl testosterone, except that a lower concentration of methyl testosterone is required for each developmental stage than with ethynyl testosterone. The lowest effective concentration for the production of growth and segment addition is 1 mg. in 14,000,000 cc. for ethynyl testosterone and 1 mg. in approximately 25,000,000 cc. with methyl testosterone. The optimal concentration for maximal growth and the formation of all differentiation areas is approximately 1 mg. in 2,000,000 cc. for ethynyl testosterone and 1 mg. in 5,000,000 cc. for methyl testosterone. A concentration higher than 1 mg. in 5,000,000 cc. produces an earlier development of the differentiation and a curtailment of growth with methyl testosterone, and with ethynyl testosterone a concentration greater than 1 mg. in 2,000,000 cc. produces the same effect. This evidence on the duration and extent of growth and segmentation at the various concentrations leads to the conclusion that the androgenic potency of methyl testosterone is from two to three times as great as that of ethynyl testosterone. This conclusion was reached also with the data for the time of appearance of the different developmental stages during treatment with the two hormones at various concentrations.

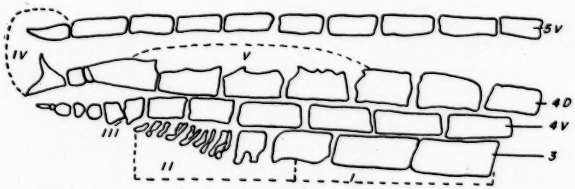
The extent to which a changing hormone concentration is responsible for the production of a regular sequence of developmental stages in the gonopodium of the normal male has been discussed. Since hormone concentration has now been shown to be related to the rate and extent of growth of each of the rays of the 3, 4, 5-ray complex in experimental females, it is appropriate to consider the role of hormone concentration in the control of total lengths of gonopodia and of single rays in the 3, 4, 5-ray complex in normal males. Some of the poeciliid fishes have long gonopodia, which reach the end of the caudal peduncle. Others have very short gonopodia. Unless these total lengths are governed by specific genetic factors, it is quite possible that they depend upon a specific rate of output of androgenic hormone by the testis. A slowly mounting output would be favorable for maximal growth, and a rapidly mounting output would soon reach a point which would be favorable for the development of differentiation areas and a termination

of growth. It is probable that the normal lengths of individual segments of rays may depend upon a regular output in specific amounts, since in the experimental females a concentration above the optimal produces abbreviated segments and any sudden increase in concentration is accompanied by a temporary period of short-segment formation (Fig. 10). The almost invariable constancy of the positions of differentiation areas and the dimensions of the areas themselves might also depend in the long run on an appropriate



9

10



11

FIG. 9.—Terminal half of rays 3, 4, and the ventral branch of ray 5 in a normal mature male gonopodium of *G. affinis*. Roman numerals indicate differentiation areas. Arabic numerals indicate rays. *D* and *V* indicate the dorsal and ventral branches, respectively.

FIG. 10.—Third ray of a female treated with ethynyl testosterone at a concentration of 1 mg./2,000,000 cc. The two segments at the right are the last formed before treatment. The first segments formed under treatment are short and irregular. Segments formed later under treatment are at the left.

FIG. 11.—Terminal part of a gonopodium formed by a female treated with ethynyl testosterone at a concentration of 1 mg./5,000 cc. Differentiation is complete. Labels are the same as in Figure 9.

hormone concentration. This is particularly true of Area IV, in which the closely associated terminal hooks are formed on different rays. The close association of the hooks depends upon the appropriate relative lengths of the rays upon which they are formed. In an experimental female a concentration just below 1 mg. in 2,000,000 cc. of ethynyl testosterone would be sufficient for maximal growth in both rays 4 and 5 but would not be high enough to promote the development of the hooks. On the other hand, if the hormone is present during development in a concentration considerably greater than 1 mg. in 2,000,000 cc., growth in ray 5 would be terminalized earlier and more extensively than in ray 4, with the result that ray 5 would be abnormally short. The terminal hooks

forming upon the ends of rays 4 and 5 would be widely separated instead of closely associated (Figs. 12 and 13). It is assumed that the same relations exist between the development of the structures of the gonopodium of the normal male and an optimal output of hormone from the testis for each stage of development.

Upon the basis of the experimental evidence it seems probable that, in the normal male, total length of gonopodium, individual lengths and segment number of rays, regularity of segments within rays, and the normal character of some of the specific structural features depend in part upon a regularly increasing output of androgenic hormone from the testis. In the last analysis this rate of output would depend upon a highly specific rate of development in the testis itself. The constancy of structural features within differentiation areas, as far as they have been outlined here, need not be dependent, therefore, only upon numerous genetic factors but in part upon a single factor for a specific developmental rate in the testis.

c) *Effect of different concentrations upon position and form of differentiated structures.*—The effect of various concentrations of hormone upon the structures developed at each of the six stages is described by stages. If the reaction during a stage is a temporary one, there will be no permanent effect upon the architecture of the fully differentiated fin; but, if the reaction is in the form of the development of additional segments or of spines, hooks, or plates, the effect is permanent. The principal objectives are to observe the types of abnormalities which are produced, the relative degree of abnormality as related to the concentration of hormone administered, and the attainment of some conclusions concerning the optimal concentration of androgenic hormone, which in the male is responsible for normal structure.

The reaction described as stage 1 and consisting of vascular changes and proliferation of the soft tissue in the region of the third, fourth, and fifth rays occurs within three or four days for all effective hormone concentrations (Fig. 1). The extent of the reaction depends upon the concentration of hormone applied. At concentrations of less than 1 mg. of ethynyl testosterone in 7,000,000 cc. of water the reaction is slight and irregular. The isolated islands of red blood cells may not develop, and the swelling of the blood vessels may be inappreciable. In these cases the axial growths and increase in segment number in rays 3, 4, and 5 occur without any visible preliminary vascular reaction. However, tissue proliferation always occurs at concentrations sufficient to stimulate growth in the 3, 4, 5-ray complex. The optimal concentration for the production of enlarged blood vessels and isolated patches of red blood cells in the tissue to the same extent as is found in the developing gonopodium of the normal male is approximately 1 mg. of ethynyl testosterone in 2,000,000 cc. or 1 mg. of methyl testosterone in 5,000,000 cc. of water. As in the normal male, the patches occur most abundantly in the regions in which active growth and differentiation are taking place throughout the active phase of gonopodial metamorphosis. In high concentrations of hormone (1 mg. of ethynyl testosterone in 10,000 cc. to 1 mg. in 500,000 cc.) the reaction is violent. Blood vessels may swell to twice their normal size and very large patches of isolated blood cells be formed. In some cases there is extensive loss of blood by hemorrhage. The soft tissues proliferate very rapidly and are so delicate that cells scarcely hold together and patches may be sloughed off. At all effective concentrations there is an enlargement of the blood vessels in the base of the fin and in the suspensory apparatus to which the fin is attached.

The effect of different concentrations upon axial growth and segmentation (stage 2) has already been described. Another reaction which occurs during stage 2 is the fusion of

the branches of rays 6, 7, and 8 and a thickening of the segments of those branches along with the segments in ray 5 at the same level. There is a beginning of the fusion of the branches at a concentration of 1 mg. of ethynyl testosterone in 10,000,000 cc. of water. Complete fusion of the branches occurs at a concentration of a little less than 1 mg. in 5,000,000, and the characteristic thickening of the segments is not produced until a specimen has been exposed to a concentration of approximately 1 mg. in 1,200,000 cc. At this concentration a medium-sized female develops precisely malelike structures in this region (Area VI). In old females, which contain numerous segments and secondary bifurcation in rays 6, 7, and 8, only the terminal twelve to twenty segments become involved. The entire area is oriented with respect to the terminal parts of the rays involved, and the securing of a close resemblance of the area in an experimental female to that in a normal male requires that the treated female be in the same stage of development as males in which the development of the area is in its initial stages. Otherwise, in the treated female, the middle portions of the rays involved will not be modified (Turner, 1941*a*, Fig. 1, *F*).

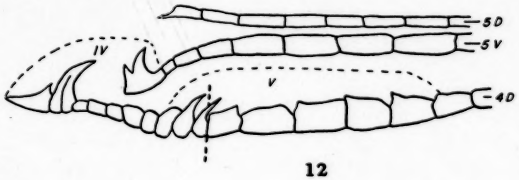
Stage 3 in the gonopodium of a normal male is marked by the first appearance of the specific structures in Area II. In treatment of females with ethynyl testosterone the stage is recognizable within seven days after the beginning of treatment at a concentration of 1 mg. in 20,000 cc. (Fig. 1) and in nineteen days at a concentration of 1 mg. in 5,000,000 cc. At a concentration of 1 mg. in 10,000,000 cc. there is no appearance of the structure. Low concentrations sufficient for the initial stage of development of the area are not high enough to maintain further development. There are three concurrent phases in the development of structures in the area—the addition of new terminal segments in ray 3, the dwindling in diameter and length of each newly added segment, and the gradual assumption of new form in each new segment, resulting in a transition from roughly rectangular plates about eighteen segments from the end to small plates with posteroventrally directed spines near the end of the ray (Fig. 9). The addition of new segments within the area is continuous throughout the process of differentiation. The concentration which is optimal for the largest number of segments is approximately 1 mg. in 2,000,000 cc. with ethynyl testosterone. At lesser concentrations the added segments fall short of the required number, and modification of the added segments is characteristic only of those which occur at some distance from the end of a normal ray. At concentrations greater than 1 mg. in 2,000,000 cc. the individual segments are somewhat shorter, and at very high concentrations the newly added segments are very short, few in number, and the plates and spines are recognizable but abnormal (Fig. 11). At no continually applied level of hormone concentration is an exact replica of the male gonopodium produced. Presumably, the most favorable conditions for the production of an almost identical structure would require a very low hormone concentration rising to a level which would be sufficient to promote the greatest growth and the addition of the largest number of segments, and at the same time not so high that abnormalities would arise in the form of short segments and poorly formed spines and plates. The rate of change in the concentration would be as important as the level of concentration.

In the gonopodia of normal males the vertical serrae on the dorsal branch of ray 4 (Area V) are associated with approximately seven segments, the posterior one of which is located four or five segments anterior to the terminal hook (Fig. 9). The position and extent of the area is only slightly variable. In the fins of experimental females the same position is assumed if the females are subjected to hormone concentrations favorable for

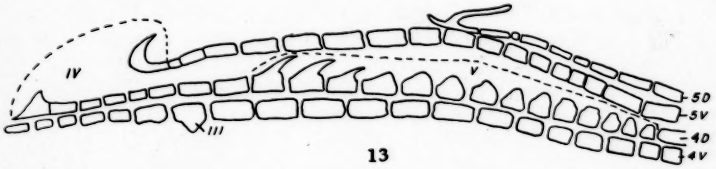
the formation of at least ten new segments in ray 4. If high concentrations are used and development of differentiation of areas is precocious, the area forms much nearer the end of the ray. Figure 11 illustrates the case of a medium-sized female which was treated with a concentration of 1 mg. of ethynyl testosterone in 5,000 cc. of water. In this case only two segments intervene between the terminal hook and the posterior segment of the Area V complex. The number of segments involved in the area will be less than normal in a female treated with a high concentration of hormone and more than normal if treated with a concentration most favorable for continued growth in the ray, approximately 1 mg. in 2,000,000 cc. of ethynyl testosterone. Five segments are involved in the area in the specimen treated with 1 mg. in 5,000 cc. (Fig. 11) and fifteen segments in a specimen treated continuously with a concentration of slightly less than 1 mg. in 2,000,000 cc. (Fig. 14). Serrae occur on the ventral branch of ray 4 in numerous species of poeciliid fishes, the number varying from four (*Flexipenis*) to thirteen (*Pseudoxiphophorus*). While these numbers are relatively constant in normal specimens of a species, the capacity to form more or less than the normal number exhibited in experimental females suggests that androgenic hormone concentration is concerned during development in the regulation of the number of segments bearing serrae. Normal form, height, and number of serrae per segment can be modified experimentally. At high concentrations serrae are found upon segments developed before treatment (Fig. 11), and they are low and often placed more than one to the segment. If a hormone concentration is used which promotes the formation of ten to twelve new segments on ray 4, the serrae will be formed upon both newly added and previously developed segments (Fig. 12). Those formed upon newly added segments are practically normal in form and number per segment, while those formed upon previously developed segments are abnormal. It is evident that hormone concentration is instrumental in experimental females in determining the position of the complex upon ray 4, the number of segments which bear serrae, and the number of serrae per segment and the form and height of the serrae. It is believed that position upon ray 4 and extent and normal form of the elements in the Area V complex depend in normal male development upon a specific androgenic hormone concentration for each stage of development and that the concentration continually increases during development.

Two principal forms of abnormality occur in Area 4 during developmental stage 5, when females are treated with hormone. The first peculiarity is a wide separation of the two terminal hooks, which in the normal male are very close together. The separation is caused by the fact that rays 4 and 5, upon which the hooks occur, respond by differential growth to high hormone concentrations and so do not reach the relative lengths which are required to bring the terminal hooks together at the time of the development of the hooks. The second type of abnormality occurs also at high concentrations and takes the form of double terminal hooks (Fig. 12). Weak terminal hooks are formed when an old female is treated with a high hormone concentration. In these cases no new segments are added to rays 4 and 5, and the hooks are formed upon the terminal segments present at the beginning of treatment (Turner, 1941*a*, Fig. 2, *F*). With high concentrations, terminal hooks are frequently formed upon rays which in normal males do not bear hooks. These occur upon the dorsal branch of ray 5 and upon one or both branches of rays 6 and 7 (Figs. 13 and 14). The same development of supernumerary terminal hooks occurs when the rays of males in late metamorphosis are cut back and regenerate under the influence of a testis which is practically mature and is producing considerable quantities of

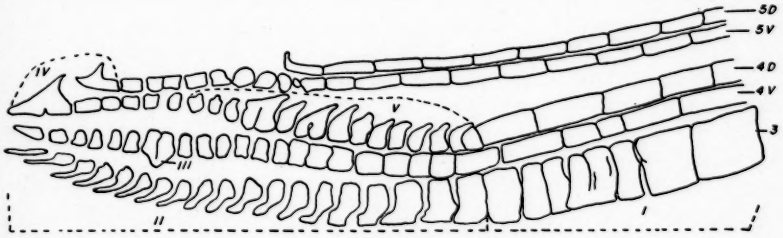
androgenic hormone (Turner, 1941*c*). The capacity to form terminal hooks is clearly demonstrated by those members which normally do not exhibit such a capacity. The occurrence may be interpreted under one of two postulates: (1) genetic determiners exist in males and females for the development of terminal hooks upon rays 6 and 7, but the hooks do not develop because normally there is not a sufficiently high concentration of



12



13



14

FIG. 12.—Terminal parts of rays 4 and 5 in a gonopodium formed by a female treated with ethynyl testosterone at a concentration of 1 mg./20,000 cc. Differentiation is complete. The labels are the same as in Figure 9. On ray 4*D* the serrae at the left of the broken line were formed upon newly added segments, those at the right upon segments formed before treatment.

FIG. 13.—Terminal parts of rays 4 and 5 in a gonopodium formed by a female treated with methyl testosterone at a concentration of 1 mg./5,000,000 cc. Terminal hook on 4*V* partially developed at this stage but completely differentiated later.

FIG. 14.—Terminal parts of rays 3, 4, and 5 in a fully differentiated gonopodium formed by a female treated with ethynyl testosterone at a concentration of 1 mg./2,000,000 cc.

androgenic hormone or (2) the field (area) within which terminal hooks develop becomes expanded under high hormone concentration and extends to the ends of the dorsal branch of ray 5 and to rays 6 and 7.

Irregularities in the structure of Area III in hormone-treated females are very common. The area does not appear at all at concentrations of less than 1 mg. in 2,000,000 cc. of ethynyl testosterone, and at this concentration the spines are likely to be low. At high

concentrations, also, its development is poor (Fig. 11). The optimum concentration for its full development is found between 1 mg. in 700,000 cc. and 1 mg. in 500,000 cc. of ethynyl testosterone.

DISCUSSION

The results obtained here with females treated with different concentrations of ethynyl and methyl testosterone clarify to some extent the relative roles played by an androgenic hormone and genetic factors in the development of the normal male gonopodium. Something of the relative influence of the antagonistic factors of growth and differentiation also become apparent.

Elsewhere (Turner, 1941*a*, *b*) the writer has postulated that the six areas arise as minor embryonic fields in the larger field of the fin as a whole and that the positions of these areas within the fin are genetically determined. It is possible that a seventh area, located near the base of the fin and involving a local thickening in rays 3 and 4, exists in *G. affinis*. It may be stated now that the receptor tissue in each of these areas possesses in the male a definite susceptibility to an androgenic hormone elaborated by the testis, since each area begins to develop only when the hormone has reached the appropriate level. It is difficult to escape the conclusion that these susceptibilities also are determined genetically. The detailed pattern developed within the areas is genetically determined to some extent. The patterns may be extensively modified as indicated by the experiments, but they are developed within wide genetic limits if they are developed at all. The constancy of the normal pattern in the mature male gonopodium, dependent in part upon the rate of hormone output by the developing testis, suggests that the relative change in amount of hormone output is constant in the development of all normal males.

The general role of the androgenic hormone may be described as that of stimulation of development in those tissues which are susceptible to its activation. In the female there are full genetic potentialities for gonopodial development in the male pattern, but they are latent until stimulated. If the androgenic hormone is present in the animal or is introduced, the concentration and the rate of increase in amount are important in the production of a normal pattern. Normal lengths of rays, lengths of segments, positions of areas, extent of areas, details of structures within the areas, and completeness of development can be modified experimentally by the application of appropriate hormone concentrations during development, and supernumerary structures never found in normal gonopodia may be induced to form by the same treatment. It is assumed that the normal development of these features in the male gonopodium depend upon (1) genetic potentialities for the isolation of the various areas or fields in definite positions, for differential susceptibilities to androgenic hormone in the receptor tissues of the fields, and for the control of the direction of development within the fields; (2) activation of the tissues by an androgenic hormone produced by the testis in increasing quantities delicately balanced with the susceptibilities of the reacting tissues.

Axial growth and segmentation in all rays of the 3, 4, 5-ray complex is maximal at a concentration of 1 mg. in 2,000,000 cc. with ethynyl testosterone treatment (Figs. 3, 4, and 5). Higher concentrations do not stimulate axial growth in a lesser degree, but they produce an earlier development of the differentiation areas, and the effect of the appearance of these areas is to terminalize axial growth. The effect is shown best at concentrations of 1 mg. in 100,000 cc. (Figs. 3, 4, and 5). If growth and differentiation are antagonistic and differentiation terminates growth, it might be expected also that a con-

centration of hormone favoring continual growth would postpone differentiation. A clear-cut demonstration of this postponement is shown in the case illustrated in Figure 13. A female was treated with methyl testosterone at a concentration of 1 mg. in 3,000,000 cc., and in due time growth was terminalized and all areas became fully developed. Stage 5, the beginning of terminal-hook development on rays 4 and 5, made its appearance in twenty-two days in the form of a hook upon the ventral branch of ray 5 and differentiation of the element was soon complete (Fig. 13). Ray 5 had ceased to grow and to add segments. At the same time ray 4 was continuing to grow and to add segments, and the terminal hook on the dorsal branch of ray 4 was not developing, although more than a sufficient concentration for its development was present, as illustrated by the development of a stage (stage 6, Area III) requiring a higher concentration. A few days later the terminal hook on the dorsal branch of ray 4 developed strongly, concurrent with the cessation of growth of the branch. The figure indicates a stage in which the hook is partially developed. A similar case, illustrated in Figure 14, is one in which a female was treated with ethynyl testosterone at a concentration of approximately 1 mg. in 2,000,000 cc. It may be noted (Fig. 4) that this concentration is the most favorable for the continuation of axial growth in ray 4, while at the same time stages 4, 5, and 6 are arising. The effect of continuing growth is most evident in Area V. The area begins to differentiate; and, if the concentration had been increased regularly, growth would have been terminalized and the area confined to six or seven segments. However, with the concentration maintained at the same level, new segments were added and differentiation proceeded along with growth, with the result that serrae were added to fifteen segments in all.

SUMMARY

1. Six successive stages in induced gonopodial development in females of *G. affinis* occur in the same order as in normal male gonopodial development.

2. The first stage is induced to develop by treatment with ethynyl testosterone at a concentration of approximately 1 mg. in 14,000,000 cc. of water (1 part in 14,000,000,000 by weight). The same result is accomplished by treating the female with methyl testosterone at a concentration of approximately 1 mg. in 25,000,000 cc. of water (1 part in 25,000,000,000 by weight).

3. Each successive stage in development requires an increase in hormone concentration for its development.

4. The optimal ethynyl testosterone concentration for the maximal amount of axial growth in rays 3, 4, and 5 and for full development of the differentiation areas is 1 mg. in 2,000,000 cc. of water. A methyl testosterone concentration of 1 mg. in 5,000,000 cc. of water produces the same result.

5. Rays 3, 4, and 5 respond differentially to growth stimulation.

6. Concentrations higher than optimal terminalize axial growth of rays 3, 4, and 5 prematurely and induce a precocious development of the differentiation areas. At concentrations optimal for continual axial growth, differentiation is postponed in the actively growing regions.

7. Segments produced under high hormone-concentration treatment are abnormally short, and structures developed within the differentiation areas are laid down within the general pattern of normal structures, but abnormality is increased as the hormone concentration is increased.

8. It is assumed that in normal male gonopodial development the direction of development and level of tissue susceptibility in each differentiation area is under genetical control and that the amount of hormone output by the developing testis controls the rate and completeness of gonopodial development.

9. In females of *G. affinis* methyl testosterone is from two to three times as potent in its androgenic activity as ethynyl testosterone.

LITERATURE CITED

BALDWIN, F. M., and GOLDIN, H. S. 1939. Effects of testosterone propionate on the female viviparous teleost *Xiphophorus helleri*. Proc. Soc. Exper. Biol. and Med., **42**:813.

BERKOWITZ, P. 1937. Effects of oestrogenic substances in *Lebistes reticulatus*. Proc. Soc. Exper. Biol. and Med., **36**:416.

BERKOWITZ, P. 1938. The effects of estrogenic substances in *Lebistes reticulatus* (Guppy). Anat. Rec., **71**:161.

EVERSOLE, W. J. 1939. The effects of androgens upon the fish (*Lebistes reticulatus*). Endocrinology, **25**: 328.

———. 1941. The effects of pregneninolone and related steroids on sexual development in fish (*Lebistes reticulatus*). *Ibid.*, **28**:603.

GROBSTEIN, C. 1940. Effects of testosterone propionate on regeneration of anal fin of adult *Platypoecilus maculatus* females. Proc. Soc. Exper. Biol. and Med., **45**:484.

REGNIER, M. T. 1937. Action des hormones sexuelles sur l'inversion du sexe chez *Xiphophorus helleri* Heckel. Compt. rend. Acad. sci., Paris, **205**:1451.

———. 1938. Contribution à l'étude de la sexualité des *Cyprinodontes vivipares* (*Xiphophorus helleri*, *Lebistes reticulatus*). Bull. biol. France-Belg., **72**:385.

ST. AMANT, LYLE. 1941. The effect of castration upon the development of the gonopodium in *Gambusia*. Unpublished Ph.D. thesis, Northwestern University.

TURNER, C. L. 1941*a*. Gonopodial characteristics produced in the anal fins of females of *Gambusia affinis affinis* by treatment with ethynyl testosterone. Biol. Bull., **80**:371.

———. 1941*b*. The morphogenesis of the gonopodium of *Gambusia affinis affinis*. Jour. Morph., **69**:161.

———. 1941*c*. Regeneration of the gonopodium of *Gambusia* during morphogenesis. Jour. Exper. Zoöl., **87**:181.

STIMULATION OF CORTICAL-CELL PROLIFERATION BY THE GROWTH HORMONE. III. EXPERIMENTS ON ALBINO RATS[1]

(Three figures)

STEPHEN ZAMENHOF
620 West One Hundred and Fifteenth Street, New York City

IN THE first paper of this series (Zamenhof, 1940) the author investigated the present possibilities of effecting such artificial and intentional changes in the architectonic of the cortex as would result in an improvement in psychical functions. It was demonstrated that the number of neurons, especially in the asensoric and amotoric areas, seems to be, next to the complexity of nerve fibers, one of the most important factors in determining the level of psychical functions. In contradistinction to the complexity of nerve fibers, which cannot be influenced artificially with the existing knowledge, the number of neurons can probably be artificially increased. This may be possible by stimulating the proliferation of prospective neurons in the early period when they have not yet lost their ability to divide. The so-called growth hormone preparation (from the eosinophile [acidophile] cells of the anterior lobe of the hypophysis) seemed to be the appropriate means of stimulating the proliferation of cells in this early period. That this opinion is justified was demonstrated in the second paper (Zamenhof, 1941) on tadpoles.

Tadpoles, of different age groups, were subjected to intraperitoneal injections of the growth hormone preparation Antuitrin G. After sectioning, the numbers of neurons and glia cells in the vicinity of the Foramen of Monro were counted. The numbers as calculated for the entire cerebral hemispheres were 44–126 per cent higher in injected animals than in the controls. These differences are statistically significant. It was therefore concluded that by means of the growth hormone it is possible to increase artificially and intentionally the number of brain cells, at least in amphibians.

The object of the present investigation, the third in the series, is to test experimentally the above theory on mammals (albino rats).

The term "growth hormone preparation," as used hereinafter, refers to the above-mentioned growth-promoting pituitary extract, regardless of whether it exists as an actual specific "growth hormone" or merely as a fortunate combination of other hormones.

LITERATURE

In man the period in which the prospective neurons still have the ability to proliferate ends in the fourth or fifth month of fetal life (Zamenhof, 1940). In rats this period is said to extend at least over twenty days after birth, being most intense in the first ten days, much less intense in the second ten days, and practically disappearing after the twentieth day (Hamilton, 1901; Allen, 1912; Sugita, 1918; Smith, 1934). Sugita (1918) is of the opinion that during this postnatal period the number of existing

[1] The author acknowledges gratefully his indebtedness to Dr. Charles A. Slanetz, of the Department of Animal Care of the College of Physicians and Surgeons, Columbia University, for the facilities provided toward the carrying-out of this work; to Dr. H. R. Halsey, of the College of Pharmacy, for the preparation of slides; and to Mr. B. Grynbaum for the technical assistance.

cortical neurons doubles, as compared with the number at birth; but he is not positive whether the new neurons are actually formed by postnatal mitoses or are simply the result of migration from the transitional and germinal layers (matrix).

Except for above publications by the author there are no publications dealing with the influence of the growth hormone on the central nervous system in this early stage. In six- to eight-month-old albino rats Rubinstein (1936) found no influence of growth hormone on brain weight; this result, however, should be anticipated, since in this age no proliferation of the neurons is still possible.

There are several publications which deal with the influence of growth hormone on hyperplasia of other tissues. Lee and Freeman (1940) demonstrated the hyperplasia of liver cells in response to the growth hormone administration. As far as the larval or fetal stage is concerned, the publications relate mostly to vertebrates lower than mammals; these publications are cited by Zamenhof (1941).

In mammals (albino rats) growth hormone was injected in pregnancy and resulted in an increased body weight of the newborn animals. Teel (1926) injected pregnant rats, from the first day of pregnancy to parturition, with 1 cc. daily of an alkaline extract of the anterior pituitary lobe containing growth hormone; the injections resulted in an increase in body weight of about 13 per cent in the fetus at term. Because of the contaminating factor in the anterior lobe extract the gestation was prolonged, and stillborn fetuses of increased weight were expelled; but in the cases when autopsy was made at term, the fetuses were alive and showed the above-mentioned increase in body weight. Hain (1932*a* and *b*) in her early experiments confirmed Teel's results about the prolongation of gestation. In further investigations (1934) she injected pregnant rats with the total of 2.5–4.0 cc. of Phyone (Wilson Laboratories); the newborn young were heavier than the controls, but in some cases growth hormone also resulted in prolongation of gestation. Sontag and Munson (1934) injected pregnant rats with Antuitrin G (Parke, Davis & Co.); the doses were 0.5 cc. daily from the eighth to the tenth day and 1 cc. from the tenth to the twenty-second day of pregnancy. The injections prolonged the gestation for one day or more; the young, removed at term, were 7.3 per cent heavier than the controls. The mortality among experimental animals was high; but many animals survived and retained their weight increase, as compared with controls, until fifty days of age, although this increase ceased to be significant. Watts (1935) administrated Phyone from the tenth day of pregnancy to delivery or from the first day to delivery. Some of the Phyone preparations were free from contaminations which prolonged gestation or interfered with normal parturition. Fetuses thus delivered at term were significantly (about 10 per cent) heavier than the controls.

There have been no experiments that deal with the injections of growth hormone during human pregnancy, although Engelbach (1932) advocated (for other purposes) such an administration. The harmlessness and the efficiency of the growth hormone injections in human infancy have been demonstrated in many cases (Putnam, Teel, and Benedict, 1928; Johnston and Maroney, 1939; Buchanan and Ballweg, 1939).

MATERIAL AND METHODS

Altogether, 385 albino rats were used in the present investigation. They were of the Sherman strain of the College of Physicians and Surgeons of Columbia University. This strain possesses many desirable qualities, including uniformity, good constitutional vigor, and fertility and is used by almost all investigators in this institution.

All the adult rats were kept in individual cages. The rats received "balanced" Rockland Mouse Diet, containing ground corn, ground hulled barley, ground hulled oats, ground whole wheat, meat scraps, powdered whole milk, alfalfa leaf meal, soy bean meal, wheat bran, wheat middlings, pulverized oats, gluten feed, fish meal, peanut flour, linseed, corn oil meal, NaCl, $CaCO_3$, and cod-liver oil. The diet was given in powdered form to mature animals during gestation and lactation and during the first fifteen days to the litter; in all other cases the diet was given in the form of pellets. The diet was supplemented twice weekly by lettuce.

The females were mated individually. The next and following days the vaginal smears were examined for sperm, and the day that sperms were found was called the first day of pregnancy. The vaginal smear was examined also later in due time for placental sign. The mature animals during pregnancy and the young were weighed daily. The young were weaned between three and four weeks of age.

The females used for breeding were five to six months old, weighed from 200 to 250 gm., and were used in the second or third pregnancy only (with a normal history of previous pregnancy). The number of young per liter was not less than 7 and not more than 13; the mean number was between 10 and 11; the mean weight of control newborn rat in the colony was 4.82 gm.; and the normal parturition in the control females was at the twenty-second or twenty-third day. These standards are regarded as "normal."

In the preliminary work 32 normal newborn rats from 9 litters were injected subcutaneously daily from the second to the twenty-first day with growth hormone preparation (Antuitrin G), since it is believed that the proliferation of neurons extends in rat over the first three weeks of life. The doses started with 0.05–0.15 cc. and increased gradually to 0.4 cc. Forty-two rats, from the same litters and having the same mean weight at birth, served as controls. This investigation yielded negative results, since injections caused a retardation in body and brain growth, as measured on animals thirty-six days old. However, control experiments, performed on other groups by injection of the same doses of neutral solution to newborn animals, showed even higher retardation; this proves that the retardation is due merely to the shock after injections and to difficulties in the assimilation of the liquid; the harmful effect was stronger than the probable effect of growth hormone. This method of administration of growth hormone after birth was therefore given up.

In the main investigations 13 normal females with normal history of first pregnancy (when used in second pregnancy) or first and second (when used in third pregnancy) were injected subcutaneously daily from the seventh to the eighteenth or twentieth day of pregnancy with 1 cc. of growth hormone preparation. Of this number, 4 females were injected with Phyone (growth hormone preparation of the Wilson Laboratories)[2] and 9 females with Antuitrin G (Parke, Davis & Co.).[3]

Since on the first 4 animals the injections of either of the preparations prolonged gestation for more than one day and since the obtaining of living fetuses at term was essential to the present investigation, in all further animals cesarean section was performed at term. Of the fetuses thus removed, 99 per cent were alive at birth, but many of them died during the first few hours. The remaining young were given to normal females in place of their own litter of the same age. This was done because the mothers

[2] Kindly furnished by Dr. David Klein, of the Wilson Laboratories, Chicago, upon the recommendation of Dr. H. B. Van Dyke.

[3] Kindly furnished by Dr. Oliver Kamm, of Parke, Davis & Co.

subjected to cesarean section were too weak to take care of the young, and besides it was desired to prevent any possible influence of growth hormone and of cesarean section on lactation. The new mothers, however, did not take care of the experimental young as well as of the normal ones and sometimes refused to take care at all; this was the cause of the death of the greater part of the experimental litter during the first twelve days of life.

For the body-weight and brain investigation of stillborn fetuses and of the young dead a few hours after delivery, the control groups were selected, consisting of normal young a few hours old, with similar weight of mothers and similar number of young in litter (Tables 1–3). Similarly, control groups of the respective age were compiled for experimental males sacrificed at about the one hundred and eighth day (Table 4) and for experimental females sacrificed at about the one hundred and twenty-fourth day (Table 5).

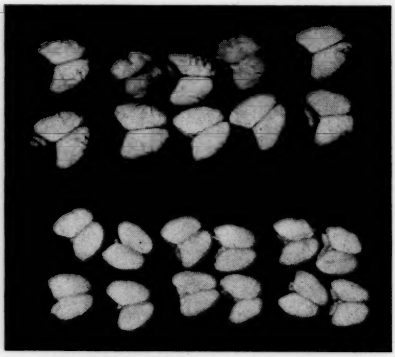


FIG. 1.—Cerebral hemispheres of one litter of experimental (upper two rows) and one litter of control (lower two rows) animals at birth. The difference in size is evident.

A part of the surviving animals, together with the control groups of exactly the same age, selected from the normal animals of the colony, were subjected to intelligence tests (maze learning) by S. Ross under the direction of Professor C. J. Warden of the Department of Psychology, Columbia University (Warden, Ross, and Zamenhof, 1942).

For the brain investigation the animals, if not stillborn, were killed with ether, weighed, and eviscerated; the bones of the skull were removed from above and the cerebral hemispheres severed from the brain stem and from the olfactory bulbs by the transverse cuts at the levels described in detail by Donaldson and Hatai (1931). Thus only cerebral hemispheres without olfactory bulbs, brain stem, or hypophysis (Fig. 1) were investigated; and weights, areas of sections, and number of cells given in this paper refer only to the cerebral hemispheres; these may differ from the data published by other investigators for the entire "brain." This method was chosen in order to have under investigation one uniform organ essential for higher psychical functions and to remove doubts as to the place where to cut off, when brains of different age are to be sectioned.

The cerebral hemispheres were then raised from the floor of the cranium and the cranial nerves clipped close to their points of emergence; then the cerebral hemispheres were removed and placed on a moist glass plate, where all three dimensions of the brain, as suggested by Sugita (1917, 1918), were measured with calipers. Immediately after this procedure the cerebral hemispheres were weighed to one-tenth of a milligram, fixed twenty-four hours in Bouin's fluid, and preserved in 70 per cent alcohol until histological sectioning. The sections of 7 μ were prepared by the paraffin method and were stained with haematoxylin and counterstained with eosin. From each animal about 48 sections were prepared, which made it possible to choose for microscopic examination

exactly the same section level in all the animals to be compared. This level (Fig. 2) was chosen in the vicinity of the Foramen of Monro.

The area of cortex was measured on the slide. Sugita (1917, 1918) suggests the introduction of corrections for changes during fixation; these corrections, however, are based on the measurements of the dimensions of fresh brain, which cannot be exact (Smith, 1934); therefore, in the present investigation, although the dimensions on fresh brain were measured, no corrections were introduced, on the assumption that if there are any changes of area value—and these changes are considered to be very slight, if, any, upon fixation in Bouin's fluid (Sugita, 1917)—they must be substantially the same in the experimental and control animals.

The limits of the cortex were chosen in the way suggested by Sugita (1917, 1918) and Smith (1934). As described by Sugita, the dorsal border was determined by the tectal

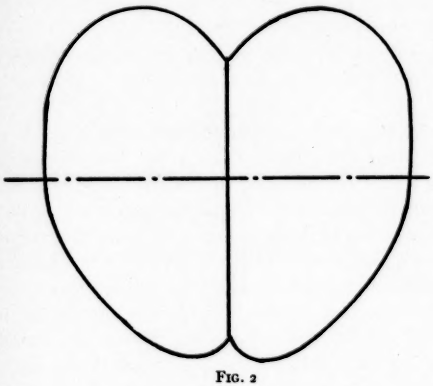


FIG. 2

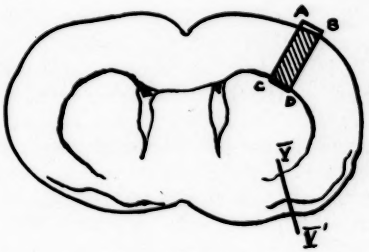


FIG. 3

FIG. 2.—Dorsal view of cerebral hemispheres, showing the level of sections.

FIG. 3.—Frontal section of cerebral hemispheres, showing the limits of the cortex as chosen in the present work, as well as the area *ABCD* examined.

border line of the corpus callosum, which lies under the tip of the cortex, and the bottom of the fissura sagittalis; the ventral border (V–V' on Fig. 3) was drawn perpendicular to the surface at the basal end of the cell group, which is found under the cortex proper, just below the region of the fissura rhinalis, lateroventral to the capsula externa.

The slides were observed with a Bausch and Lomb microscope under a low magnification (×120) in the case of mature animals and high (×440) in the case of newborn animals. The counting of the number of cells, as well as the calculation of areas of the sections, was performed by means of a Spencer reticule 5 mm. square, ruled into one hundred squares, inserted in the eyepiece of the microscope. Sugita (1918) in his work calculated the cells in five adjoining squares along the cortical band in two layers only (lamina pyramidalis and lamina zonalis). Although Sugita's results have been generally accepted (Donaldson, 1924), in the present work the method of counting cells in two layers only has not been considered reliable enough, since the layers are not sharply defined and since two layers only cannot represent the characteristic of the entire cortex. In the present work, therefore, the number of cells in a band ten squares in width, going

throughout the entire thickness of the cortex (Fig. 3) has been calculated. This method takes much more time than that of Sugita, but the results are more accurate. Altogether, about 790,000 cells were counted. The counting was performed by means of a hand tally counter. The cells in each section were counted two or three times, and the mean of these counts was determined. The cells were counted in the locality *ABCD* (Fig. 3), corresponding to Locality VII in Sugita's work (1918).

It should be pointed out that in the newborn brains it is not possible to tell apart the prospective neurons and the prospective glia cells. Since the object of this investigation was merely to compare the number of cells in the experimental and control animals and not the investigation of the exact number of cells, all the cells in the newborn brains were counted (except, of course, cells of capillary walls, leucocytes, lymphocytes, etc.). In the mature brains all neuron and glia cells of the nuclei diameter larger than 8 μ were counted. The cells were counted by their nuclei, and therefore the parts without nuclei were not counted in both experimental and control animals. These methods, which were adapted also by Sugita (1918), are quite satisfactory for comparison of experimental animals with controls, although not exact if the absolute number of cells is to be accurately determined.

The calculation of experimental data has been performed as follows. The area of one square in the eyepiece for both magnifications used has been calculated. The number of cells in the band ten squares wide throughout the thickness of the cortex, divided by 10 and by the thickness of the cortex (expressed in number of squares) and multiplied by the area of one square (in mm.), yields the density of cells per square millimeter of section (or rather per volume of 0.007 cu. mm., since the sections are all 7 μ thick). The thickness of the cortex in millimeters and the area of the cortex in both hemispheres on the slide, in square millimeters, are calculated from the number of squares as observed. The number of cells per section is calculated as usual by multiplying the density of cells per square millimeter by the area of the cortex (in sq. mm.) on both hemispheres.

By comparing the results for experimental animals with those for the controls, the increase in weight of cerebral hemispheres, in cortex thickness, in cell density, in area of cortex on slide, and in number of cells in the experimental animals over the controls has been calculated. This increase is expressed in percentage of normal (control) values.

To obtain the values for the entire volume of the cortex it was assumed that the increase in area and in density of cells is similar in all sections or in all three dimensions. The ratio of section areas in experimental and in control animals raised to the $3/2$ power represents the ratio of volumes of the cortex in experimental and in control animals; from the ratio of volumes the increase in volume was calculated. The ratio of volumes multiplied by the ratio of previously calculated densities per volume of 0.007 cu. mm. yields the ratio of number of cells in the entire volume of the cortex; from the latter, again, the increase (in percentages) in number of cells in the entire volume of cortex in experimental animals over controls was calculated.

The significance of the mean values was investigated by means of the statistical "x-test" for large groups and the "t-test" for small groups, as described in the textbooks by Fisher (1938), Fisher and Yates (1938), and Mainland (1938).

RESULTS AND DISCUSSION

1. *Newborn rats.*—The results relating to newborn rats are given in Tables 1–3.

The weight at birth (Table 1) in the animals whose mother was injected in pregnancy

was found to be 5.72 ± 0.0671 gm., which is 18.7 per cent higher than in the controls (4.82 ± 0.0414 gm.). This increase is higher than the corresponding data in the literature quoted before. The increase is statistically significant.

The animals, especially when stillborn, were often as heavy as 8.8 gm.; similar results (newborn as heavy as 8.6 gm.) were found by Watts (1935).

TABLE 1

NEWBORN ANIMALS

Group	Number of Animals	Number of Litters	Mean Litter size	Mean Weight of Mother (In Gm.)	Sex		Body Weight at Birth ± Standard Error (In Gm.)	Probability	Increase in Percentage of Control (Normal) Value
					♂	♀			
Control.....	122	12	10.2	224	60	62	4.82 ± 0.0414	Less than 0.00001 (significant)	18.7
Experimental..	127	13	10.6	226	58	69	5.72 ± 0.0671		

TABLE 2

NEWBORN ANIMALS

Group No.	Sex		Pregnancy No.	Number in Litter	Mean Body Weight ± Standard Error (In Gm.)	Probability	Increase in Percentage of Control (Normal) Value
	♂	♀					
5 (Control).....	4	3	II	7	4.57 ± 0.0815	Less than 0.001 (significant)	12.28
5 (Exper.).....	3	7	III	10	5.131 ± 0.0675		
7 (Control).....	7	3	II	10	5.605 ± 0.199	Less than 0.001 (significant)	25.42
7 (Exper.).....	4	8	III	12	7.03 ± 0.188		
8 (Control).....	4	6	II	10	5.04 ± 0.1255	Less than 0.002 (significant)	13.26
8 (Exper.).....	5	5	III	10	5.709 ± 0.1255		

In regard to Table 1 it should be noted that the ratio of males to females in the control group was slightly higher, and the mean number of fetuses in the litter slightly lower, than in the experimental animals; all these factors indicate that the mean weight of young should be higher in control groups than in the experimental.

Since the best control animals are those born from the same mother, Table 2 was compiled to compare the experimental animals (groups 5 Exper., 7 Exper., 8 Exper.) in third pregnancy with the control animals (groups 5 Control, 7 Control, 8 Control)

in second pregnancy from the same mothers, respectively. The results are similar to those given in Table 1; the mean increase in weight is 17 per cent, which is also statistically significant.

The experiments, as far as the body weight at birth is concerned, confirm, therefore, the previous findings, namely, that the growth hormone administrated in pregnancy is able to stimulate the somatic growth of the fetus.

TABLE 3

NEWBORN ANIMALS

Group	Number of Animals	Number of Litters	Mean Cerebral Hemisphere Weight ± Standard Error (In Mg.)	Mean Area of Cortex on Slide ± Standard Error (In Sq. Mm.)	Mean Cortex Thickness ± Standard Error (In Mm.)	Mean Cell Density ± Standard Error	Mean Number of Cells per Section ± Standard Error (In Ten Thousands)
Control.....	65	6	146.0 ± 1.2	6.06 ±0.089	0.775 ±0.0118	5,540 ± 104	3.355 ±0.0656
Experimental....	69	11	198.5 ± 3.36	8.64 ±0.125	0.938 ±0.0143	6,050 ± 112.5	5.230 ±0.0939

PROBABILITIES

For Cerebral Hemisphere Weight	For Area of Cortex on Slide	For Cortex Thickness	For Cell Density	For Number of Cells in Section
Less than 0.00001 (significant)	Less than 0.00001 (significant)	Less than 0.00001 (significant)	Less than 0.001 (significant)	Less than 0.00001 (significant)

INCREASES

(In Percentage of Normal [Control] Values)

In Cerebral Hemisphere Weight	In Area of Cortex on Slide	In Volume of Cortex	In Thickness of Cortex	In Cell Density	In Number of Cells per Section on Slide	In Number of Cells in Entire Volume of Cerebral Cortex
36	42.6	70.4	21	9.27	56	86.5

New in the present investigations are findings concerning macroscopic and microscopic comparison of the cerebral hemispheres (Table 3) in the experimental and control animals.

As can be seen from Table 3, the mean weight of cerebral hemispheres in experimental animals was 198.5 ± 3.36 mg. and in controls 146.0 ± 1.2 mg. This increase amounts to 36 per cent and is statistically significant. In some animals, especially when stillborn, the cerebral hemispheres were as heavy as 263 mg.

Figure 1 represents cerebral hemispheres of one litter of experimental animals (upper row) and one litter of control animals (lower row). The differences in size are evident.

Although in mature animals the increased weight of cerebral hemispheres does not necessarily mean a higher number of cerebral cells, yet in the newborn brain, because of the crowded conditions, heavier cerebral hemispheres correspond in general to higher number of cells. The microscopic investigation confirmed the above results. The mean

TABLE 4

ADULT ANIMALS—MALE GROUP

(Mean Age: 108 Days)

Group	Number of Animals	Number of Litters	Mean Litter Size	Mean Body Weight (In Gm.)	Mean Weight of Cerebral Hemisphere (In Gm.)	Mean Area of Cortex on Slide (In Sq. Mm.)	Mean Cortex Thickness (In Mm.)	Mean Cell Density	Mean Number of Cells per Section (In Ten Thousands)
Control.....	13	3	10	233.9 ± 10.1	1.3115 ±0.0203	28.6 ± 0.868	1.594 ±0.077	710 ± 23	2.02 ±0.064
Experimental..	9	4	10	254.8 ± 12.133	1.324 ±0.0244	30.1 ± 1.04	1.634 ±0.09	906.23 ± 27.6	2.697 ±0.07

PROBABILITIES

For Area of Cortex on Slide	For Cortex Thickness	For Cell Density	For Number of Cells in Section
Nonsignificant	Nonsignificant	Less than 0.001 (significant)	Less than 0.001 (significant)

INCREASES

(In Percentage of Normal [Control] Values)

In Area of Cortex on Section	In Volume of Cortex	In Thickness of Cortex	In Cell Density	In Number of Cells per Section on Slide	In Number of Cells in Entire Volume of Cerebral Cortex
5.25	8.0	2.5	27.6	33.5	38

thickness of cortex in experimental animals was found to be 21 per cent higher, the area of cortex section 42.6 per cent higher, the volume of cortex 70.4 per cent higher, the number of cells per section 56 per cent higher, and the number of cells in the entire cortical volume 86.5 per cent higher (Table 3). All these results are statistically significant. Thus the action of growth hormone resulted in a strong stimulation of cortical-cell proliferation and in an accentuated hyperplasia of these cells. This is especially evident by comparing the cell density and the total number of cells in experimental and control animals.

The comparison of Tables 1 and 3 reveals the following: The influence of the growth

hormone on hyperplasia of cortical cells is much more accentuated than on hyperplasia of other cells (compare increase in total weight of the body). This result conforms with the author's opinion as expressed in his first paper (Zamenhof, 1940)—that cells such as neurons, which at an early stage lose their ability to divide because of early differentiation, should be much more susceptible to the proliferation stimuli (growth hormone) acting within the specified time than are the other cells with a long proliferation period

TABLE 5

ADULT ANIMALS—FEMALE GROUP

(Mean Age: 124 Days)

Group	Number of Animals	Number of Litters	Mean Litter Size	Mean Body Weight (In Gm.)	Mean Cerebral Hemisphere Weight (In Gm.)	Mean Area of Cortex on Slide (In Sq. Mm.)	Mean Cortex Thickness (In Mm.)	Mean Cell Density	Mean Number of Cells per Section (In Ten Thousands)
Control.....	10	2	10	174.9 ± 7.1	1.238 ±0.0207	25.4 ± 0.95	1.488 ±0.0279	765 ± 33.4	1.94 ±0.133
Experimental..	9	4	10	185.6 ± 7.95	1.237 ±0.0218	29.2 ± 1.0	1.552 ±0.0265	878 ± 35.2	2.56 ±0.141

PROBABILITIES

For Area of Cortex on Slide	For Cortex Thickness	For Cell Density	For Number of Cells in Section
0.012 (significant)	Nonsignificant	0.03 (significant)	0.005 (significant)

INCREASES

(In Percentage of Normal [Control] Values)

In Area of Cortex on Section	In Volume of Cortex	In Thickness of Cortex	In Cell Density	In Number of Cells per Section on Slide	In Number of Cells in Entire Volume of Cerebral Cortex
14.5	22.5	4.3	14.8	31.7	40.6

to the stimuli acting within the same specified time, because the period of stimulation is relatively longer in the former cells than in the latter. In higher mammals, including man, this may make possible, as is shown in the rat, an artificial and considerable increase in number of neurons without excessive increase in body size of the fetus, which otherwise could impair parturition.

2. *Mature rats.*—The next groups of animals were investigated at the age of one hundred and eight days (Table 4, males only) and one hundred and twenty-four days (Table 5, females only). Although, as mentioned before, in the first days the neurons in

rat still have a certain ability to proliferate and in this period (postnatally) the experimental animals did not receive any injections of growth hormone, yet the experimental animals retained their prenatal preponderance (in density and in total number of cortical cells) over the nontreated controls. In both groups one hundred and eight and one hundred and twenty-four days old the increase in cell density was 14.8–27.6 per cent and the increase in number of cells per volume was 38.0–40.6 per cent. Both these increases are statistically significant.

In females one hundred and twenty-four days old, also, the increase in section area of the cortex (14.5 per cent) and in volume of the cortex (22.5 per cent) are statistically significant.

Further comparison of tables reveals that the differences in body weight, cerebral-hemisphere weight, and dimensions of the cortex between experimental and control animals gradually cease to be statistically significant and finally almost vanish. This result means that the experimental animals tend to assume normal body and macroscopically normal cortex, while retaining their high preponderance in the number of cortical cells (and probably in some other types of cells which lose their ability to proliferate early).

CONCLUSION

From the above results the following conclusions can be reached:

1. Growth hormone preparation, when injected into pregnant mammals, is able to increase significantly the density and the total number of cortical cells in the offspring at maturity, as compared with the normal animals.

2. Measured at birth, also, the body weight, cerebral-hemisphere weight, thickness of cortex area of the cortex section, and volume of cortex are significantly increased as compared with the normal animals.

SUMMARY

Three hundred and eighty-five albino rats were used in further investigation of the theory, outlined in the author's previous papers, that by means of the growth hormone preparation it may be possible to stimulate artificially the proliferation of prospective cerebral neurons in mammals, in an attempt to improve the level of the psychical function of the animal.

The rats were subjected in pregnancy to subcutaneous injections of two growth hormone preparations, Antuitrin G and Phyone. The offspring investigated at birth showed an increase of 18.7 per cent in body weight, 36 per cent in cerebral-hemisphere weight, 21 per cent in thickness of the cerebral cortex, 70.4 per cent in volume of the cortex, 9.27 per cent in density of cells, and 86.5 per cent in number of cells per volume of cortex, as compared with the controls. All these increases are statistically significant.

A certain number of animals reached maturity and showed an increase of 14.8–27.6 per cent in cell density and 38.0–40.6 per cent in total number of cortical cells, as compared with the controls. These increases are statistically significant.

LITERATURE CITED

ALLEN, E. 1912. The cessation of mitosis in the central nervous system of the albino rat. Jour. Comp. Neurol., **22**:547.

BUCHANAN, J. A., and BALLWEG, H. A. 1939. A case of pituitary dwarfism treated with Antuitrin-G. Endocrinology, **24**:565.

DONALDSON, H. H. 1924. The rat. Memoirs of the Wistar Inst., No. 6. Philadelphia: Wistar Institute.
DONALDSON, H. H., and HATAI, S. 1931. On the weight of the parts of the brain and on the percentage of water in them according to brain weight and to age, in albino and in wild Norway rats. Jour. Comp. Neurol., **53**:263.
ENGELBACH, W. 1932. The growth hormone. Endocrinology, **16**:1.
FISHER, R. A. 1938. Statistical methods for research workers. Edinburgh: Oliver & Boyd.
FISHER, R. A., and YATES, F. 1938. Statistical tables for biological, agricultural and medical research. Edinburgh: Oliver & Boyd.
HAIN, A. M. 1932*a*. Increase in weight of the mother and of the foetus during pregnancy (rat). Quart. Jour. Exper. Physiol., **22**:71.
———. 1932*b*. The physiology of pregnancy in the rat. *Ibid.*, p. 249.
———. 1934. The physiology of pregnancy in the rat. *Ibid.*, **24**:101.
HAMILTON, A. 1901. The division of differentiated cells in the central nervous system of the white rat. Jour. Comp. Neurol., **11**:297.
JOHNSTON, J. A., and MARONEY, J. W. 1939. Observation on nitrogen and calcium balances as affected by growth and gonadotropic hormones administrated for short periods to growing children. Endocrinology, **25**:199.
LEE, M., and FREEMAN, W. 1940. Liver growth in rats treated with anterior pituitary growth hormone. Endocrinology, **26**:493.
MAINLAND, D. 1938. The treatment of clinical and laboratory data. Edinburgh: Oliver & Boyd.
PUTNAM, T. J.; TEEL, H. M.; and BENEDICT, E. B. 1928. The preparation of a sterile, active extract from the anterior lobe of the hypophysis. Amer. Jour. Physiol., **84**:157.
RUBINSTEIN, H. S. 1936. The effect of the growth hormone upon the brain and brain weight–body weight relations. Jour. Comp. Neurol., **64**:469.
SMITH, C. G. 1934. The volume of the neocortex of the albino rat and the changes it undergoes with age after birth. Jour. Comp. Neurol., **60**:319.
SONTAG, L. W., and MUNSON, P. L. 1934. The effect on the weight of the offspring of administration of Antuitrin G to the pregnant rat. Amer. Jour. Physiol., **108**:593.
SUGITA, N. 1917. Comparative studies on the growth of the cerebral cortex. Jour. Comp. Neurol., **28**:511.
———. 1918. Comparative studies on the growth of the cerebral cortex. *Ibid.*, **29**:61.
TEEL, H. M. 1926. The effects of injecting anterior hypophysial fluid on the course of gestation in the rat. Amer. Jour. Physiol., **79**:170.
WARDEN, C. J.; ROSS, S.; and ZAMENHOF, S. 1942. The effect of artificial changes in the brain of maze-learning in the white rat. Science, **95**:414.
WATTS, R. M. 1935. The effect of administration of preparation of growth hormone of the anterior lobe of the pituitary upon gestation and the weight of the newborn (albino rats). Amer. Jour. Obst. and Gynec., **30**:174.
ZAMENHOF, S. 1940. On present possibilities of increasing the higher functions of the cortex through artificial changes in its architectonic. Lancaster: Science Press Printing Co. Pp. 28.
———. 1941. Stimulation of the proliferation of neurons by the growth hormone. II. Experiments on tadpoles. Growth, **5**:123.

STUDIES ON THE HYDROGEN-ION CONCENTRATION OF INSECT BLOOD AND THEIR BEARING ON IN VITRO CYTOLOGICAL TECHNIQUE[1]

ROBERT D. BOCHE AND JOHN B. BUCK
University of Pennsylvania and University of Rochester

INTRODUCTION

IN DISPUTES over the visible structure of cytological objects it is customary to refer, if possible, to what can be seen in the "living" or "normal" cell. This sort of evidence has long been used, for example, in investigations on mitochondria, Golgi bodies, and spindle fibers; and recently it has been appealed to in studies on the perplexing structure of the giant salivary gland chromosomes of dipteran larvae. In the last instance, however, the reports based on "living" material agree no better than those from fixed preparations. Thus Bauer (1935) and Painter and Griffin (1937) maintained that chromosomes were visible in "living" salivary gland nuclei of *Chironomus* and *Simulium*, respectively, whereas Doyle and Metz (1935*a*, *b*) and Caspersson (1936) maintained that they were not visible in *Sciara* and *Drosophila*, respectively.[2] Since several species were used in these investigations, the reported differences might represent interspecific variation. This idea is supported, in part, by the observations of Buck (1939) in which *Chironomus*, *Drosophila*, *Sciara*, and *Simulium* were studied by an *in vivo* technique, which obviated the need for dissection and its consequent possibilities of injury and were found to differ in the visibility of their chromosomes. On the other hand, Barigozzi (1938) worked on the same species as Bauer (*Chironomus Thummi*) and reported that the chromosomes were sometimes visible and sometimes not. This indicates either a locality difference, as suggested by Barigozzi, or a difference in the techniques used to examine the "living" chromosomes.

The usual method for *in vitro* study of "normal" or "living" giant chromosomes has been to excise the salivary glands and examine them either in a salt solution of more or less arbitrary composition or in the body fluid ("blood" or "haemolymph") of the larva, either with or without a covering of paraffin oil.[2] Similar methods are adequate for some material, particularly where cell division or growth can be used as a criterion of normality. This has been amply demonstrated, for example, by the studies of Chambers (1925) and Bělař (1929) on living grasshopper spermatocytes and by tissue-culture work.[3] Nevertheless, the discordant reports cited earlier (and others) indicate strongly that *in vitro* methods have been inadequate, as applied to giant chromosomes.

[1] This work was made possible by a Rockefeller Foundation grant to Dr. C. W. Metz and was done mainly at the Department of Embryology, Carnegie Institution of Washington, Baltimore. We are greatly indebted to Dr. Metz for facilities, advice, and encouragement and to various colleagues for valuable aid with the manuscript.

[2] In the literature there are twenty or more equally contradictory descriptions of the appearance and properties of "living" salivary gland chromosomes. These reports and the techniques employed will be considered in detail in a forthcoming publication.

[3] This is contested, however, by Baumgartner and Payne (1931), who maintained that all *in vitro* techniques introduce cytological abnormalities.

It has been shown *in vitro* by Doyle and Metz (1935*a*, *b*), by Shinke (1937), by Guareschi (1939), and by Stefanelli (1939) and *in vivo* by Buck and Boche (1938, and in preparation) that relatively slight changes in the osmotic pressure of the fluid surrounding the salivary gland cause very marked changes in the visible structure of the chromosomes. It is likely, therefore, that the salt solutions used in the investigations referred to earlier were sufficiently diverse in composition and concentration to account for a good deal of the reported variation in the appearance of the chromosomes. Blood, however, is isosmotic with, and neutral to, the gland cells originally; so diverse results obtained in work in which this ambient was used might be due to *changes* occurring in the blood subsequent to dissection. A most likely sort of change is in the hydrogen-ion concentration ($[H^+]$), owing to chemical processes in the blood or the tissue.

This investigation was made, first, to ascertain whether changes in $[H^+]$ occur in standing exteriorized insect blood and, second, to measure the $[H^+]$ of certain insect bloods as a preliminary to preparing properly buffered blood substitutes for *in vitro* work on salivary gland chromosomes.

MATERIALS AND METHODS

Mature larvae of *Chironomus tentans*, collected in the field, and of *Sciara coprophila* and *Drosophila melanogaster* from pedigreed stock cultures were used. From 1 to 100 larvae were sacrificed for each measurement, according to the amount of blood necessary. The larvae were first rinsed in distilled water to decrease external contamination and briefly drained on filter paper; then put into the depression of a Pyrex-glass culture slide, where their haemocoels were opened with a pair of steel needles. The resulting pool of blood was taken up in a clean, dry capillary pipette and transferred to the electrode. The time elapsing between the start of dissection and the measurement varied between 1 and 3 minutes. In rupturing the larval body wall, particular care was taken not to puncture or lacerate any other structure, especially the gut, several parts of which are known to be markedly different in $[H^+]$ from the blood (Crozier, 1924; Boche and Buck, 1938).

Some preliminary work was done with indicator dyes (Boche and Buck, 1938), but, in the main, two types of electrodes were used. The first was a modification of the microquinhydrone electrode of Pierce and Montgomery (1935), used with a sensitive potentiometer. This method is applicable to extremely small amounts of fluid; but, as quinhydrone electrodes are subject to salt and protein errors, which are not inherent in glass electrodes, most of the measurements were made with the second type, a capillary glass electrode similar to that supplied commercially with the Beckman pH-meter. The capillaries were made from Corning 015 glass, had capacities ranging from 5 $mm.^3$ to 20 $mm.^3$ (estimated), and were used with the Coleman pH-meter and a Ag-AgCl half-cell. These electrodes were simple to make and remained stable for dozens of readings. Their accuracy was checked against a standard series of seven phosphate buffers, ranging from pH 4.6 to pH 8.3, and the determinations were found to differ less than 0.1 pH unit from duplicate measurements made with a standard macroglass electrode. A rough estimate of the experimental error of the method was obtained by making successive measurements on a standard buffer of pH 6.89: In 17 measurements, extending over 3 hours, the mean value pH 6.88 ± 0.009 (S.E.) was obtained. A minor difficulty, sometimes encountered because of the high surface-volume ratios of the capillaries, was a steady downward drift in $[H^+]$ of liquids standing in the electrode. This drift was due to alkali dissolving from the walls of the capillary, rather than due to changes in asym-

metry potential, because the readings returned to their original values if the electrode was drained and refilled with a fresh sample. With buffered solutions, however, the drift was so slow that rapid manipulation yielded reliable values, and consequently all readings were made as quickly as possible after filling the electrode. The capillary was rinsed thoroughly with distilled water between readings, and the asymmetry potential was checked before and after each reading. Potassium acid phthalate, M/15, was used as a primary standard. Its $[H^+]$ was taken as pH 3.98 at 25° C.

RESULTS

Table 1 gives the results of measurements on *Drosophila* and *Sciara* blood made with both types of electrode. This table indicates that the blood of both species is slightly alkaline. The fact that there was good agreement between the results obtained with the

TABLE 1*

$[H^+]$ OF TISSUE-FREE BLOOD OF *Drosophila* AND *Sciara*

Each measurement on *Drosophila* was made on blood from 50–100 larvae; each on *Sciara*, on blood from 4–100. All larvae used in a given measurement came from the same culture. Only one measurement was made on each sample of blood. Usually only one measurement was made on larvae from a given culture.

Drosophila (pH VALUES)		*Sciara* (pH VALUES)	
Glass	Quinhydrone	Glass	Quinhydrone
7.09	7.21	7.25	7.34
7.11	6.92	7.12	7.28
7.03	7.18	7.14	7.48
7.15	7.18	7.06	7.46
7.07	.....	7.03	7.32
7.13	.....	7.15	.....
Mean 7.10	7.12	Mean 7.12	7.38

* No measurement reported in any table in this paper is repeated in any other table.

quinhydrone and glass electrodes with *Drosophila* but not with *Sciara* suggests that there is some species difference in blood composition which affects the electrodes differently. Since so many larvae, all the same age, were used for each determination, individual variations undoubtedly canceled out; but too few measurements were made to decide whether the variation between lots of larvae was a true intraspecific variation or was due simply to experimental error.

Table 2 presents the results of 53 measurements on blood from 9 different lots of *Chironomus* larvae. It shows that the $[H^+]$ of *Chironomus* blood averaged about pH 7.5 and that it sometimes differed significantly in different lots of larvae. *Chironomus* larvae yield so much more blood than *Drosophila* or *Sciara* that they were used in all subsequent experiments.

Evidence was presented in the introduction suggesting that changes in $[H^+]$ might

occur in blood subsequent to dissection. To test this possibility, measurements were made at intervals on successive aliquots of pools of *Chironomus* blood. The results of from 3 to 5 such successive measurements on each of 5 samples of blood are presented in Table 3. This table shows that *Chironomus* blood exposed to air became more alkaline,

TABLE 2

$[H^+]$ OF TISSUE-FREE *Chironomus* BLOOD

Only one measurement was made on a given sample of blood. All measurements reported in one horizontal column were from samples from the same lot of larvae. Capillary glass electrodes were used.

Number of Samples Measured	Number of Larvae per Measurement	Mean pH and Its Standard Error
17.....	2–3	7.57±0.02
3.....	1–2	7.23
9.....	3	7.32±0.04
2.....	2	7.48
6.....	2–9	7.57±0.03
12.....	1–11	7.45±0.02
2.....	10	7.72
1.....	8	7.48
1.....	9	7.66
Total 53	Arithmetic mean=7.48 weighted=7.49	

TABLE 3

CHANGE IN $[H^+]$ IN 5 SAMPLES OF TISSUE-FREE *Chironomus* BLOOD WITH TIME OF EXPOSURE TO AIR

Horizontal rows contain successive measurements on aliquots of one original sample. T = total elapsed minutes between start of dissection and measurement.

Number of Larvae per Measurement	T	pH	T	pH	T	pH	T	pH	T	pH	T	pH
10.....	1	7.62	17	7.80	..	....	39	7.93	60	8.04	77	8.07
10.....	4	7.40	21	7.57	..	....	45	7.68	..	....	..	....
7.....	2	7.55	16	7.69	28	7.70	44	7.79	58	7.94	..	....
9.....	3	7.59	14	7.69	27	7.79	..	....	..	....	..	....
11.....	2	7.52	18	7.65	34	7.73	45	7.81	..	....	..	....
Mean 9.4	2.4	7.53	17	7.68	30	7.74	43	7.80	59	7.99	..	....

to the extent of about 0.4 pH units in 1 hour. This finding agrees in principle with that of Bishop (1923), who found an increase in alkalinity of approximately 0.5 pH units in *Apis* blood equilibrated with hydrogen for ½ hour; but it is at variance with that of Pepper, Donaldson, and Hastings (1941), who found no change in *Anobrus* blood held at 3°–4° C. Since Pyrex glass is nearly insoluble in water, it is unlikely that the decrease

in $[H^+]$ was due to alkali dissolving from the slide. It is more probable that the drift was due to loss of CO_2 from the blood into the surrounding air.

Since the dissection-to-measurement interval varied unavoidably, it might be supposed that differences in the extent of the alkaline drift during this interval might account for some of the apparent intraspecific variability seen in Tables 2 and 3. However, the increase was slow; and, since the measurements were made immediately after dissection, the drift is believed not to have been an important source of error in the above measurements.

TABLE 4

RELATION BETWEEN TIME AND $[H^+]$ IN 3 SAMPLES OF TISSUE-FREE *Chironomus* BLOOD COVERED WITH PARAFFIN OIL

Symbols and explanation as in Table 3.

Number of Larvae per Measurement	*T*	pH	*T*	pH	*T*	pH	*T*	pH	*T*	pH	*T*	pH
	Oil Layer 1 Mm. Thick											
9	3	7.63	23	7.73	46	7.73	69	7.77	..	....	94	7.88
9	2	7.64	22	7.67	43	7.71	62	7.74	80	7.83	96	8.01
	Oil Layer 5 Mm. Thick											
8	2	7.48	18	7.54	39	7.50	58	7.49	79	7.52	96	7.58

TABLE 5

EFFECT OF CONTACT WITH LARVAL TISSUE FRAGMENTS ON $[H^+]$ OF 2 SAMPLES OF *Chironomus* BLOOD

Symbols and explanation as in Table 3.

Number of Larvae per Measurement	*T*	pH	*T*	pH	*T*	pH	*T*	pH	*T*	pH	*T*	pH
10	2	7.72	12	7.63	24	7.55	36	7.52	48	7.53	..	....
10	2	7.72	13	7.64	24	7.56	39	7.50	53	7.50	65	7.47

Kubie (1927) reported that the rate of diffusion of CO_2 in paraffin oil is low. It is therefore possible that CO_2 loss could be diminished by collecting the blood under oil, although CO_2 is more soluble in paraffin oil than in water. Accordingly, larvae were dissected under 1- and 5-mm. layers of Nujol; the blood was freed of larval tissue; and aliquots were removed at intervals from under the oil, transferred to the glass electrode, and measured. The results of from 5 to 6 such successive measurements on each of 3 samples of *Chironomus* blood are presented in Table 4. With due allowance for the small number of experiments the table indicates that a 1-mm. layer of oil only slightly

TABLE 6

[H^+] OF THE BLOOD OF VARIOUS INSECTS

G = glass electrode; Q = quinhydrone electrode; H = hydrogen electrode; C = colorimetric.

Order and Species	Observer	Method	pH Range	Average pH
Coleoptera:				
Aromia moschata	Kocian and Špaček	Q	.....	6.15
A. moschata		C	6.2 –6.3	.....
Calosoma inquisitor		C	6.6 –6.8	6.7
C. sychophauta		C	6.6 –6.7	.....
C. sychophauta		Q	.....	6.65
Cantharus nigra		C	6.0 –6.3	6.2
Carabus cancellatus		Q	6.95–7.05	.....
C. cancellatus		C	6.6 –7.3	7.0
C. violaceus		C	6.9 –7.1	7.0
Cerambyx cerdo		C	6.2 –6.5	6.3
Cetonia aurata		C	6.5 –6.9	6.7
Coccinella septempunctata		C	6.9 –7.3	7.1
C. septempunctata		Q	.....	7.25
Cotinis nitida	Fink	C	.....	7.3
Dorcus parallelopipedus	Kocian and Špaček	C	6.2 –6.3	.....
Dytiscus marginalis		C	6.8 –7.3	7.1
D. marginalis		Q	.....	7.12
Epilachna borealis	Fink	C	.....	5.9
Geotrupes stercorarius	Kocian and Špaček	C	6.2 –6.4	6.3
Hydrophilus piceus		C	6.7 –7.0	6.8
H. piceus		Q	.....	6.55
Leptinotarsa decemlineata	Fink	C	5.9 –6.8	.....
Lucanus cervus	Kocian and Špaček	C	6.7 –6.9	6.8
L. cervus		Q	.....	6.75
Lytta vesicatoria		C	6.7 –6.8	6.75
Meloe violacea		Q	.....	7.2
M. violacea		C	6.9 –7.3	7.2
Melolontha vulgaris		Q	6.25–6.31	.....
M. vulgaris		C	6.0 –6.6	6.25
Necrophorus vespertilio		C	6.5 –6.8	6.7
Popillia japonica	Ludwig	H	6.23–7.36	.....
Prionus coriarius	Kocian and Špaček	C	6.3 –6.5	6.4
Procrustes coriarius		C	7.2 –7.3	.....
Silpha atrata		C	6.8 –6.9	6.8
S. quadripunctata		C	6.7 –6.9	6.8
Tenebrio molitor		C	6.2 –7.3	.....
T. molitor		Q	6.35–7.15	6.7
Diptera:				
Hylemyia cilicrura	Fink	C	5.9 –7.1	.....
Musca domestica	Glaser	C	7.2 –7.6	.....
Hemiptera:				
Anasa tristis	Fink	C	6.6 –6.8	.....
Hymenoptera:				
Apis mellifica	Bishop	H	6.77–6.93	6.83
A. mellifica	Hoskins and Harrison	C	.....	6.70
A. mellifica		G	6.63–6.85	6.70
Sceliphron caementarium	Booker	Q	6.3 –6.8	.....

TABLE 6—*Continued*

Order and Species	Observer	Method	pH Range	Average pH
Lepidoptera:				
Ancylis comptana	Fink	C	5.9 –6.8	.....
Bombyx mori	Jameson and Atkins	C	6.6 –6.8	.....
B. mori	Glaser	C	6.4 –7.2	.....
B. mori	Demjanowski *et al.*	Q	6.6 –6.8	.....
B. mori	Ongaro	Q	6.82–6.86	.....
B. mori	Epstein	H	6.22–6.63	6.5
Deilephila euphorbiae	Heller and Moklowska	Q	6.36–6.64	.....
Galleria mellonella	Taylor and Birnie	G	5.53–6.68	.....
G. mellonella	Taylor, Birnie, *et al.*	G	5.8 –6.4	.....
Heliothis obsoleta	Craig and Clark	G	6.66–7.3	.....
Malacosoma americanum	Glaser	C	6.4 –7.4	.....
Pieris brassicae	Brecher	H	6.50–6.77	6.6
P. rapae	Fink	C	5.9 –6.4	.....
P. rapae	Craig and Clark	G	6.76–7.56	.....
Prodenia eridanea	Babers	Q	6.40–6.67	6.53
Spilosoma menthastrii	Beljajew*	?	6.0 –6.6	.....
Vanessa urticae	Brecher	H	6.5 –6.77	6.6
Orthoptera:				
Anobrus simplex	Pepper *et al.*	G	.....	7.23
Arphia xanthoptera	Bodine (1925)	C	6.7 –7.0	6.83
Chortophaga australior		C	.....	6.8
C. viridifasciata		C	.....	6.8
C. viridifasciata	Bodine (1926)	H	6.43–7.05	6.79
Conocephalus ensiger	Bodine (1925)	C	.....	6.7
Dicromorpha viridis		C	6.4 –6.9	6.7
Dissosteira carolina		C	6.8 –6.9	6.85
Encoptolophus sordidus		C	6.7 –6.9	6.77
Melanoplus bivittatus		C	6.0 –6.7	6.35
M. differentialis		C	6.4 –7.0	6.81
M. differentialis	Bodine (1926)	H	6.42–6.98	6.68
M. differentialis	Glaser	C	7.2 –7.6	.....
M. femur-rubrum	Bodine (1925)	C	6.7 –6.86	6.79
M. femur-rubrum	Bodine (1926)	H	6.4 –6.8	6.73
M. scudderi	Bodine (1925)	C	6.6 –6.9	6.8
Microcentrum retinervis		C	.....	6.3
Orphula pelidina		C	.....	6.75
Periplaneta americana	Glaser	C	7.5 –8.0	.....
Romalea microptera	Bodine (1926)	H	6.4 –6.8	6.73
Scudderia furcata	Bodine (1925)	C	.....	6.7
Schistocerca americana		C	.....	6.6
Spharagemon bolli		C	.....	6.8
Trimerotropis maritima		C	.....	6.8
Trichoptera:				
Anabolia nervosa	Krey	Q	6.94–7.0	.....
Halesus digitatus		Q	6.87–6.94	.....
Limnophilus flavicornis		Q	7.04–7.18	.....
Molanna angustata		Q	6.99–7.04	.....
Phryganea obsoleta		Q	6.80–6.98	.....
P. striata		Q	6.71–6.81	.....

* From Epstein.

retarded the drift of the tissue-free *Chironomus* blood toward greater alkalinity but that a 5-mm. layer considerably retarded this drift. Presumably, in the latter instance, loss of CO_2 was limited by the low speed of diffusion, once the oil layer adjacent to the blood was saturated.

All the measurements thus far reported were on blood removed immediately from contact with larval remains. Since it seemed likely that respiration and necrotic changes in the tissues would affect the $[H^+]$ of the blood if this precaution were not taken, the possibility was tested by leaving blood in contact with fragments of larvae and measuring aliquots at intervals. The results are presented in Table 5. It shows that *Chironomus* blood in contact with tissue fragments became increasingly acid with time, the change averaging 0.2 pH units in the first half-hour. Since the blood was also in contact with air, this increase in acidity took place in spite of the opposing tendency to become alkaline due to loss of CO_2. The acidification was presumably due to accumulation of acid metabolites from the tissues.

DISCUSSION

Since no determinations of blood $[H^+]$ in the genera *Sciara*, *Drosophila*, and *Chironomus* have been made previously, the measurements reported above will be considered briefly, in relation to those reported for other insects, before discussing the more important subject of the changes which supervene in standing blood.

The literature on insect blood is fairly extensive and has never been thoroughly reviewed. For lack of space, the present survey is limited to the data presented in Table 6, which summarizes the main results reported and which may serve as a guide to the original papers. Most of the work involved too few measurements to permit an assessment of their standard errors, etc., or to permit valid comparisons to be drawn. Table 6 omits references dealing with the $[H^+]$ of parts of the gut or other tissues, unless they also include results on blood, and also observations of a qualitative nature, such as those describing the reaction of litmus to blood. The table includes 69 species, distributed among 7 orders, as follows: Coleoptera, 27; Diptera, 2; Hemiptera, 1; Hymenoptera, 2; Lepidoptera, 11; Orthoptera, 20; Trichoptera, 6. Considering Table 6 as a whole, the data indicate that (*a*), although there are noteworthy interspecific differences, insect blood tends to be slightly acid (only 11 species out of 69 are reported to have an average pH value exceeding 7.0, and even of these, 3 are acid according to other reports); (*b*) there is often marked intraspecific variation; and (*c*) in species studied in more than one investigation (e.g., *Apis*, *Bombyx*, *Melanoplus*) there is considerable diversity in the reported $[H^+]$. Although it is likely that some of the apparent variation seen in Table 6 is due to the use of insects of different age, sex, and developmental stage, it is also probable, first, that insects have not as precise a control of blood $[H^+]$ as mammals and, second, that different methods for measuring $[H^+]$ give considerably different results.

Sciara, *Drosophila*, and *Chironomus* show the same order of variability in blood $[H^+]$ as the insects listed in Table 6, and exhibit no special peculiarities, save in being among the minority having alkaline blood. *Chironomus* particularly, with an average pH value of 7.5, is at the extreme alkaline limit among insects thus far investigated. In this connection it is interesting to note that Rapkine and Wurmser (1926) estimated the intracellular $[H^+]$ of *Chironomus* to be more alkaline than pH 7.4.

If the changes in $[H^+]$ observed in standing *Chironomus* blood hold for other species, they have a very significant bearing on the reliability of the standard *in vitro* technique

for observing "living" insect cells. It is common knowledge that many of the striking visible changes observed in chromosomes during cytological fixation are correlated with changes in [H^+], and it has been shown also (Lewis, 1923; Sakamura, 1927; Zeiger, 1935; Buck and Boche, 1938; etc.) that similar changes can be induced reversibly in "living" cells by temporary and relatively minor alterations in the [H^+] of the extracellular ambient. It is accordingly clear that changes in blood [H^+] are a likely cause of some of the reported differences in the appearance of "living" giant chromosomes. Moreover, there is no reason to doubt that [H^+] changes alter the chromosomes of other cells and, indeed, the cytoplasmic constituents as well. Therefore, where a true and reproducible picture of normal cellular conditions in insects is desired, it seems imperative that physiological salines, if used, be buffered or that blood, if used, be free from tissue fragments and kept from contact with air.

SUMMARY

1. Measurements by capillary glass electrode of the hydrogen-ion concentration of the larval blood of 3 species of Diptera give the following average results: *D. melanogaster*, pH 7.10; *Sciara coprophila*, pH 7.12; *Chironomus tentans*, pH 7.48. Microquinhydrone electrodes, considered somewhat less reliable, give the values pH 7.12 for *Drosophila* and pH 7.38 for *Sciara*. Critiques of the methods and the variability of blood hydrogen-ion concentration are presented.

2. *Chironomus* blood in contact with air becomes markedly more alkaline with time, probably because of escape of CO_2. The rate of change is not greatly altered by covering the blood with a layer of paraffin oil 1 mm. thick, but it is decreased considerably by a layer 5 mm. thick.

3. *Chironomus* blood in contact with air becomes steadily more acid if it is also in contact with larval tissue fragments. This is due presumably to accumulation of acid metabolites from the tissue.

4. The facts summarized in conclusions 2 and 3, together with the well-known marked effects of changes in hydrogen-ion concentration on cells, show that the conventional *in vitro* method of examining "living" or fresh insect tissues is likely to be grossly unreliable unless the ambient used is properly buffered and kept from contact with air and tissue fragments.

5. A table summarizing most of the existing literature on the hydrogen-ion concentration of insect blood is included.

LITERATURE CITED

BABERS, FRANK H. 1938. An analysis of the blood of the sixth-instar southern army worm (*Prodenia eridania*). Jour. Agric. Res., **57**:697–706.

BARIGOZZI, CLAUDIO. 1938. Esperienze di microdissezione sui cromosomi delle ghiandole salivari di *Chironomus* sp. Arch. f. exper. Zellforsch., **22**:190–95.

BAUER, HANS. 1935. Der Aufbau der Chromosomen aus den Speicheldrüsen von *Chironomus Thummi* Kiefer. Zeitschr. f. Zellforsch., **23**:280–313.

BAUMGARTNER, W. J., and PAYNE, M. ANTHONY. 1931. "Intravitam" technic used in studies on the living cells of grasshoppers. Jour. Exper. Zoöl., **59**:359–93.

BĚLAŘ, K. 1929. Beiträge zur Kausalanalyse der Mitose. II. Untersuchungen an der Spermatocyten von *Chorthippus* (*Stenobothrus*) *lineatus* Panz. Arch. f. Entw'mech., **118**:359–484.

BISHOP, GEORGE H. 1923. Body fluid of the honey bee larva. I. Osmotic pressure, specific gravity, pH, O_2 capacity, CO_2 capacity, and buffer value, and their changes with larval activity and metamorphosis. Jour. Biol. Chem., **58**:543–65.

BOCHE, ROBERT D., and BUCK, JOHN B. 1938. Hydrogen-ion concentration of insect blood. Anat. Rec., Suppl., **72**:108–9.

BODINE, JOSEPH HALL. 1925. Physiology of the Orthoptera. Hydrogen ion concentration of the blood and alimentary tract of certain Orthoptera (grasshoppers). Biol. Bull., **48**:79–82.

———. 1926. Hydrogen ion concentration in the blood of certain insects (Orthoptera). *Ibid.*, **51**:363–69.

BOOKER, W. M. 1932. Studies on the pH changes of body fluids during metamorphosis in an insect, *Sceliphron caementarium* (Hymenoptera). Anat. Rec., Suppl., **54**:47.

BRECHER, LEONORE. 1925. Physiko-chemische und chemische Untersuchungen am Raupen- und Puppenblute (*Pieris brassicae*, *Vanessa urticae*). Zeitschr. f. vergl. Physiol., **2**:691–713.

BUCK, JOHN B. 1939. Structure of living salivary gland chromosomes. Genetics, **24**:96.

BUCK, JOHN B., and BOCHE, ROBERT D. 1938. Some properties of living chromosomes. Collecting Net, **13**:201–3, and Anat. Rec., Suppl., **72**:73–74.

CASPERSSON, TORBJÖRN. 1936. Über den chemischen Aufbau der Strukturen des Zellkernes. Skand. arch. f. physiol., **73**: Suppl. 8. Pp. 151; see esp. p. 132.

CHAMBERS, ROBERT. 1925. Etudes de microdissection. IV. Les structures mitochondriales et nucléaires dans les cellules germinales mâles chez la sauterelle. Cellule, **35**:107–19.

CRAIG, RODERICK, and CLARK, JOHN R. 1938. The hydrogen-ion concentration and buffer value of the blood of larvae of *Pieris rapae* (L) and *Heliothis obsoleta* (F). Jour. Econ. Entom., **31**:51–54.

CROZIER, W. J. 1924. Hydrogen ion concentrations within the alimentary tract of insects. Jour. Gen. Physiol., **6**:289–93.

DEMJANOWSKI, S.; GALZOWA, R.; and ROSHDESTWENSKA, W. 1932. Wahre Azidität der Hämolymphe von *Bombyx mori* L. Biochem. Zeitschr., **247**:386–405.

DOYLE, W. L., and METZ., C. W. 1935*a*. Observations on the structure of living salivary gland chromosomes in *Sciara*. Proc. Natl. Acad. Sci., **21**:75–78.

———. 1935*b*. Structure of the chromosomes in the salivary gland cells in *Sciara* (Diptera). Biol. Bull., **69**:126–35.

EPSTEIN, W. 1930. Zur Frage über die Determination des Voltinismus beim Seidenspinner (*Sericaria mori* L.). Russkii zoologicheskii zhurnal, **10**:77–89.

FINK, DAVID E. 1925. Metabolism during embryonic and metamorphic development of insects. Jour. Gen. Physiol., **7**:527–43.

GLASER, R. W. 1925. Hydrogen-ion concentrations in the blood of insects. Jour. Gen. Physiol., **7**:599–602.

GUARESCHI, CELSO. 1939. La Morfologia della cromatina delle ghiandole salivari di *Chironomus plumosus* in rapporto ad esperienze chimico-fisische. Boll. di zool., **10**:109–14.

HELLER, J., and MOKLOWSKA, A. 1930. Über die Zusammensetzung des Raupenblutes bei *Deilephila euphorbiae* und deren Veränderungen im Verlauf der Metamorphose. Biochem. Zeitschr., **219**:473–89.

HOSKINS, W. M., and HARRISON, A. S. 1934. The buffering power of the contents of the ventriculus of the honey bee and its effect upon the toxicity of arsenic. Jour. Econ. Entom., **27**:924–42.

JAMESON, ALEXANDER PRINGLE, and ATKINS, WILLIAM RINGROSE GELSTON. 1921. On the physiology of the silkworm. Biochem. Jour. **15**:209–12.

KOCIAN, V., and ŠPAČEK, M. 1934. Die Bestimmung der Wasserstoffionenkonzentration der Körperflüssigkeit von Coleopteren. Zool. Jahrb., **54**:180–90.

KREY, JOHANNES. 1937. Untersuchungen zur Ökologie und Physiologie der Trichopterenlarven. Zool. Jahrb., **58**:201–24.

KUBIE, LAWRENCE S. 1927. The solubility of O_2, CO_2, and N_2 in mineral oil, and the transfer of carbon dioxide from oil to air. Jour. Biol. Chem., **72**:545–48.

LEWIS, MARGARET REED. 1923. Reversible gelation in living cells. Johns Hopkins Hosp. Bull., **34**:373–79.

LUDWIG, D. 1934. Studies on the metamorphosis of the Japanese beetle *Popillia japonica* Newman. Changes in the pH of the blood. Ann. Entom. Soc. Amer., **27**:429–34.

ONGARO, D. 1929–30. La Concentrazione idrogenionica dell'uovo del Filugello. Atti Reale ist. Veneto sci., **89**:145–54.

PAINTER, THEOPHILUS S., and GRIFFIN, ALLEN B. 1937. The structure and the development of the salivary gland chromosomes of *Simulium*. Genetics, **22**:612–33.

PEPPER, J. H.; DONALDSON, F. T.; and HASTINGS, ELLSWORTH. 1941. Buffering capacity and composition of the blood serum and regurgitated digestive juices of the Mormon cricket (*Anobrus simplex* Hald.). Physiol. Zoöl., **14**:470–75.

PIERCE, J. A., and MONTGOMERY, HUGH. 1935. A microquinhydrone electrode: its application to the determination of the pH of glomerular urine of *Necturus*. Jour. Biol. Chem., **110**:763–75.

RAPKINE, LOUIS, and WURMSER, RENÉ. 1926. Sur le potential de réduction du noyau et les oxydations cellulaires. Compt. rend. Soc. biol. France, **94**:989–90.

SAKAMURA, T. 1927. Chromosomenforschung an frischen Material. Protoplasma, **1**:537–65.

SHINKE, NAMIO. 1937. An experimental study on the structure of living nuclei in the resting stage. Cytologia, F. J., pp. 449–63.

STEFANELLI, ALBERTO. 1939. Sul comportamento dei colloidi nucleari delle ghiandole salivari di *Chironomus plumosus*. Esperienze di micromanipolazione combinate con esperienze chimico-fisische. Boll. di zool., **10**:149–62.

TAYLOR, I. R., and BIRNIE, JAMES H. 1932. Hydrogen-ion activity changes in *Galleria mellonella* during metamorphosis as determined by a glass electrode with micro vessel. Anat. Rec., Suppl., **54**:39.

TAYLOR, I. R.; BIRNIE, J. H.; MITCHELL, P. H.; and SOLINGER, J. L. 1934. Hydrogen-ion activity changes in *Galleria mellonella* during metamorphosis as determined by a glass electrode with micro vessel. Physiol. Zoöl., **7**:593–99.

ZEIGER, K. 1935. Zum Problem der vitalen Struktur des Zellkernes. Zeitschr. f. Zellforsch., **22**:607–32.

STUDIES ON VIGOR IN THE ROTIFER HYDATINA SENTA[1, 2, 3]

ELMER W. HERTEL
University of Nebraska

THIS investigation deals with the effects of parthenogenetic reproduction, bisexual inbreeding, and crossbreeding on vigor in four unrelated strains of the rotifer *Hydatina senta*. Increase in vigor in both plants and animals often follows the process of crossbreeding and has been a familiar phenomenon to both plant and animal hybridizers for more than two centuries. A history of the investigations on vigor will not be reviewed, since excellent bibliographies on this subject have been given by previous writers, such as East and Hayes (1912) and East and Jones (1919).

The studies which comprise this investigation on the phenomenon of vigor, as it is expressed in *H. senta*, were carried on from November, 1938, to July, 1941. The fundamental purposes of the investigation were to gain a better understanding of the manner in which the phenomenon of vigor is expressed in the animal kingdom and to study the various possibilities of *H. senta* as an experimental animal in genetical research.

MATERIALS AND CULTURE METHODS

Vigor may be expressed in a variety of ways in different organisms. Increase in size, fecundity, rate of growth, and resistance to disease are a few of such expressions. In this investigation on *Hydatina* the main criterion used to measure vigor was the size of the parthenogenetic family. Shull (1912) determined six distinct means for measuring vigor in *Hydatina*, namely, the size of the family of parthenogenetic females, the number of days required for the animal to reach maturity, the number of eggs laid per day, the size of the family of sexual females, the proportion of instances in which the first daughter did not become the parent of the new generation, and the difficulty encountered in rearing given races. In the present studies careful observations were made on such factors as difficulty in rearing, resistance to bacterial infection, the size of the families of sexual females as determined by the number of fertilized eggs deposited, and the length of life of the individual. As far as measuring vigor is concerned, there is a high correlation among all of these factors, with the possible exception of the length of life and the size of the family of parthenogenetic females.

Hydatina senta presents many advantages for research on the phenomenon of vigor. The animal multiplies rapidly, is easily cultured in the laboratory, its sexes can be controlled to facilitate inbreeding and crossbreeding, and it reproduces both sexually and

[1] Submitted to the faculty of the Graduate School of the University of Nebraska in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

[2] "Studies from the Zoölogical Laboratories," University of Nebraska, No. 213.

[3] The writer wishes to thank particularly Professor D. D. Whitney, of the deaprtment of zoölogy, under whose direction this investigation was carried on and whose assistance and suggestions have been invaluable.

This investigation was aided by a grant from the University of Nebraska Research Council, to which the author wishes to make a grateful acknowledgment.

parthenogenetically, which is particularly advantageous. Bisexual reproduction permits inbreeding and crossbreeding, while parthenogenetic reproduction makes possible a very accurate observation of the exact degree of vigor expressed by the animal. As was previously stated, vigor was measured in these studies by the size of the parthenogenetic family. Families of successive parthenogenetic generations have the same genetic constitution, for in the parthenogenetic reproduction of females only one polar body is formed, and there is no reduction in the number of chromosomes in the egg during maturation. In consequence of this fact, after bisexual inbreeding or crossbreeding has been performed, the resulting progeny can be raised through several parthenogenetic generations, and the exact degree of vigor lost or gained can be recorded. This character of *Hydatina* is a distinct advantage, so far as these studies are concerned, over those organisms which reproduce only bisexually.

All the rotifers used in these studies were cultured under conditions kept as uniform as possible. The culture solution consisted of 70 cc. of rain water, 30 cc. of spring water, and 20 drops of concentrated "stable tea." The "stable tea" was prepared by placing 1,000 gm. of fresh horse manure in 1,200 cc. of tap water, which was then sterilized in an autoclave for 60 minutes under 15 pounds pressure. The combination of spring water, rain water, and "stable tea" had a pH of 7.2. After making several studies on the effects of H-ion concentration on the rate of reproduction, it seemed that 7.2 was optimum. A buffer solution was consequently added to the culture solution to retain a constant pH throughout the life of the rotifer. To every 100 cc. of culture solution were added 10 cc. of a buffer solution having a pH of 7.2. The buffer solution was a combination of 50 cc. 0.2 N KH_2PO_4 and 35 cc. 0.2 N NaOH, diluted to 200 cc. (Mathews, 1931). The necessity of retaining a constant pH throughout the life of the rotifer arises from the fact that a change in H-ion concentration immediately modifies the rotifer's rate of reproduction. It is, of course, necessary to keep the same pH raising successive parthenogenetic and bisexual generations in order that the results of studies on the rate of reproduction will be consistent. The use of buffer solutions for the purpose described above was developed in the present investigation.

The culture solution in which the rotifers were raised was maintained at a temperature of from 16° to 18° C. throughout all of the experimental work. This was accomplished by culturing the rotifers in a double-walled water cabinet, between the walls of which was kept flowing a stream of tap water with a temperature of from 16° to 18° C.

A culture of the colorless flagellate *Polytoma* was used as food for the rotifers during parthenogenetic reproduction. Ferris (1932) has described the method for culturing this protozoan animal.

Polytoma were prepared for feeding the rotifers by removing the film from the surface of the culture with a sterilized spoon, centifuging, and washing twice with the regular culture solution in which the rotifers were raised. One cc. of the washed and concentrated *Polytoma* was diluted with 15 cc. of the culture solution. One drop of this *Polytoma* food material was placed daily in each Syracuse watch glass containing 8 cc. of the culture solution and a female rotifer. The culture solution in the watch glass was not changed during the time the rotifer was producing her family of daughters.

To obtain male-producing females, *Chlamydomonas* was fed to the rotifers with the regular *Polytoma* diet. Whitney (1914) found that a diet of *Chlamydomonas*, in contrast to a pure diet of *Polytoma*, would produce as high as 80 per cent male-producing individuals in a strain of *Hydatina*. In this investigation also the use of *Chlamydomonas* was

a consistent means of producing male-producing females for fertilization in inbreeding and crossbreeding studies.

Chlamydomonas was raised in 200-cc. glass tumblers placed in a special cabinet which allowed sunlight to reach the cultures. The cabinet was constructed so that it could be placed outside of a south window during the day and brought inside during the night or during periods of unfavorable weather. The temperature was controlled as in the cabinet for culturing rotifers. During the day, when the cabinet was exposed to direct sunlight, the temperature would rise considerably, depending upon the temperature outside of the cabinet. However, the temperature was never permitted to rise above 35° C., because a temperature in excess of 35° C. is fatal.

The medium used for culturing *Chlamydomonas* consisted of two capsules of Wilson's desiccated liver, one-eighth of an Armour's beef bouillon cube, 250 mg. of desiccated cabbage, and 250 mg. of NaCl. The above constituents were placed in 800 cc. of tap water, boiled, and later cooled. *Chlamydomonas* thrived best when the solution was changed every 3 days by decanting about two-thirds of the old solution and adding new medium. The organisms became abundant at the surface of the solution and at the edges of the glass tumblers. They could be removed in large numbers in a few drops of the culture solution by means of a small pipette. Care was taken in raising *Chlamydomonas* to keep out the larger species of protozoans which would eat and deplete the cultures.

DESCRIPTION OF THE EXPERIMENTS

Four separate strains of *Hydatina* were selected for the work here described. The strains were the original stock cultures and are designated as strains I, II, III, and IV. An individual of a strain, with its parthenogenetic progeny, is considered a family. Several successive parthenogenetic families, originating from one individual female, is considered a line. The history of the strains indicates that they were unrelated. Strain I was taken in the fall of 1938 from a stock culture of rotifers which were kept in the laboratory. Strain II was the progeny of a female which was hatched from a fertilized egg which had been stored in a battery jar of old culture water since 1936. Strains III and IV were taken from wild cultures which were collected from ponds in the vicinity of Lincoln, Nebraska. The locality of the ponds and the time of collection would indicate that these two strains were probably not related to each other.

The rotifers were raised in Syracuse watch glasses containing 8 cc. of the culture solution. In determining the size of the parthenogenetic families, the young were removed from the dishes at 12-hour intervals. Records were kept which showed the date at which the generation was started, the date at which reproduction ceased, and the number of young removed at 12-hour intervals. Five parthenogenetic generations were raised and recorded from each individual.

The index of vigor for a given line was determined not on the basis of the size of a single generation but from the average number of individuals produced in five successive generations. Likewise, the range, mean, and standard deviation of each strain were computed from the average of five successive generations. The standard error, rather than the probable error, of the mean and of the standard deviation was computed. Also, the homogeneity of the strain was tested by the method of analysis of variance. Tables 1, 2, 3, and 4 show the index of vigor for the four strains at the time this investigation was started. Ten individuals from each strain were considered a fair sample of the strain,

since all reproduction was parthenogenetic from the time the individual rotifer was selected to represent the line.

Whitney (1907) and Ferris (1932) have described the life-cycle of *H. senta*. To understand the process of inbreeding and crossbreeding, this life-cycle should be well in mind.

When *Hydatina* is fed pure cultures of a colorless protozoan, *Polytoma*, only parthenogenetic females are produced, with an occasional exception. Upon adding the green flagellate *Chlamydomonas* to the diet, many male-producing individuals will result, which makes possible controlled inbreeding and crossbreeding of races and lines. Inbreeding of the lines was accomplished by feeding *Chlamydomonas* to a few sister individuals in a Syracuse watch glass. A small amount of *Polytoma* was fed with the *Chlamydomonas*. In a few days males would appear which would fertilize the eggs of the mictic females also present in the watch glasses. This is a close-relationship mating, since all the individuals mated were the parthenogenetic progeny of one female. The fertilized eggs were removed by means of a small pipette and with the aid of a binocular. The eggs were dried in a desiccator containing CaCl crystals for at least 3 days to promote hatching within a short time. Drying in ordinary laboratory conditions will promote hatching, but the use of the desiccator speeds up the process considerably. After removing the eggs from the desiccator, regular culture water was poured upon them. *Polytoma* was added to the culture solution while the eggs were hatching, to supply food for the young. As soon as the young female rotifers were observed after hatching, they were removed, and each was placed in a separate watch glass. Each female started a new line, and its vigor was then determined in the manner already mentioned.

Each of the strains was inbred bisexually for three generations. The purpose of this inbreeding was to study the effects of inbreeding and to determine whether pure homozygous lines for the factors involved in the controlling of vigor could be obtained. After the parental strains were in bred, in the manner already described, twenty fertilized eggs were hatched, and a record was made of the number of their progeny. This was the S_1, inbred, generation. One of the weakest lines from each strain was then inbred to become the S_2 generation. Each line which was selected for inbreeding was allowed to reproduce parthenogenetically until enough individuals were on hand to accomplish bisexual inbreeding to the next generation. In the S_2 generation thirty eggs were hatched in each strain and the progeny counted in each of the thirty lines which started from a fertilized egg. The weakest lines from the S_2 generation of each strain were then inbred bisexually to the S_3 generation. Thirty eggs were hatched from each inbreeding, and the index of vigor was determined. The inbreeding of the strongest lines from each strain is not as yet complete. The effects of inbreeding on the four strains will be discussed later.

A series of crossbreeding experiments were also carried on in this investigation. Crossbreeding between two strains was accomplished in the following manner: A female of one strain (strain A) was placed in a watch glass with culture solution and fed *Chlamydomonas* with some *Polytoma*. The young daughters were isolated, each in a dish, and fed *Polytoma*. Many of the daughters would produce males within 36–48 hours. About 36 hours after the parental females from strain A were isolated, one female from the other strain (strain B) was isolated in a watch glass with culture solution and was likewise fed *Chlamydomonas* and *Polytoma*. As the daughters were hatching from the female of strain B, the males from strain A were placed in the watch glass with them. As the

young females were hatched from strain B, they were impregnated with sperm from the males of strain A. This impregnation with sperm can be accomplished only when the daughters are very young. As the young impregnated females became larger, they were removed to separate watch glasses and fed *Polytoma*. After they reached maturity, they deposited fertilized eggs. The fertilized eggs were dried in a desiccator and later hatched by the addition of culture solution in the manner already described.

Strains I and II were crossed reciprocally at the beginning of the investigation. Ten eggs were hatched from this cross, and the vigor of the lines was determined. Strains III and IV were also crossed, but not reciprocally. Thirty eggs were hatched from this cross, and the vigor of the lines was determined.

After each of the strains were inbred for three generations, their progeny were crossed. A weak line from strain I was crossed with a weak line from strain III. In the same manner a weak line from strain II was crossed with a weak line from strain IV. The purpose of crossing the lines after the strains had been inbred for three generations was to determine to what extent vigor would return to the progenies of these crosses.

In addition to the experiments on the effects of inbreeding and crossbreeding, several additional studies were made. To determine whether the members of one parthenogenetic family displayed the same degree of vigor, the first twenty and the last fifteen members of one family were raised through five parthenogenetic generations, and the number of their progency were recorded. Although this did not include every member of a single family, the first twenty and the last fifteen members is a fair representation of a single family.

Observations were made and recorded in regard to the number of parthenogenetic and fertilized eggs deposited by weak and strong females and the percentage of these eggs which hatched. Of 762 parthenogenetic female eggs which were laid by females in weak lines of *Hydatina*, 581, or 76.2 per cent, hatched. In strong lines a total of 1,860 eggs were observed, with 1,494, or 80.3 per cent, hatching. These results represent an average of 78.25 per cent hatching in both lines. The percentage of fertilized eggs which hatched both in weak and in strong lines was low in comparison to the percentage of parthenogenetic eggs which hatched. Out of 450 fertilized eggs which were under observation in weak and strong lines, an average of only 9.1 per cent hatched in both lines, with slight variation between the weak and strong. In the male-producing females the percentage of eggs which hatched was similar to that of the parthenogenetic female eggs. Out of 1,034 eggs which were observed in both weak and strong lines, 71.4 per cent hatched into male rotifers. From these observations on the number of eggs laid and the number which hatched, one must conclude that the degree of vigor of the rotifer affects only the number of eggs laid and not the viability of the eggs.

PARTHENOGENETIC REPRODUCTION

In parthenogenetic reproduction in the rotifer *H. senta* there is a development of a new individual, either male or female, from a germ cell or egg without fertilization. Parthenogenetic reproduction is common in the species. The writer wishes to emphasize that, in all the experiments in this investigation which involved the determining of the exact degree of vigor of any line, the progeny of five parthenogenetic generations was counted. The average of the number produced in the five generations was then considered the index of vigor of that line. Since environmental conditions play such an im-

portant part in the rate of reproduction within a line, the number of progeny produced in one generation is not a reliable figure. If, however, the number of progeny produced in five generations does not vary to any great extent within the limits of experimental error, one can be reasonably sure that the true index of vigor has been determined. To procure successive generations with approximately the same number of progeny, one must be extremely careful in counting the young, and environmental conditions must

TABLE 1

SHOWING NUMBER OF YOUNG FEMALES PRODUCED BY TEN LINES FROM STRAIN I

The first female of all lines was selected at random from among the immediate parthenogenetic progeny of the one mother which was selected to represent the strain.*

	Serial Number of Strain										Generation
	1	2	3	4	5	6	7	8	9	10	
Number of young females produced in each generation	38	37	34	31	40	40	40	40	37	40	1
	44	42	36	46	38	45	37	47	46	40	2
	47	45	40	46	41	42	41	43	48	45	3
	41	42	43	40	38	42	45	40	40	40	4
	42	36	37	46	38	40	45	45	37	44	5
Av.	42.4	40.4	38	41.8	39	41.8	41.6	43	41.6	41.8	.....

Mean, 41.14±0.49. Standard deviation, 1.55±0.33. Range from 38 to 43
Test by analysis of variance shows no significance between line means but significance between generations

* Table 1 is given in detail to show the method of recording the number of progeny produced in five parthenogenetic generations. Given below is a table showing the test by analysis of variance for Table 1. The tables which are to follow will give only a summary of the results of that particular study.

ANALYSIS OF VARIANCE FOR STRAIN HOMOGENEITY IN TABLE 1

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square
Total	49	656.02	.....
Generations	4	198.52	49.63*
Strains	9	108.82	12.09
Error	36	348.68	9.69

* Highly significant.

be uniform. As will be noted later, weak lines show considerably more variations in successive parthenogenetic generations than do the strong. This is undoubtedly due to the difficulty of culturing the weak individuals.

At the beginning of the investigation the vigor of the four strains of rotifers was determined by means of parthenogenetic reproduction. Each strain was started from one female rotifer. This female was allowed to reproduce parthenogenetically until enough individuals were on hand to begin investigation. Ten females were then selected at random, and each was placed in a watch glass with culture solution. They were fed

Polytoma, and the youth were removed at 12-hour intervals. The individuals selected for strain I had an average index of vigor of 41.14 ± 0.49 (Table 1). The standard deviation was 1.55 ± 0.33, and the range was from 38 to 43.[4] Since the standard deviation is so low, one might be tempted to assume that the race is homozygous for the factors involved in the control of vigor; however, this condition was due to the fact that all individuals were the progeny of the same female. The rotifers making up the general cultures from which the one individual was selected representing the strain were considered vigorous. Considerable variation occurred between individuals, indicating that they were probably heterozygous for the factors involved in vigor. This is borne out by the results obtained from inbreeding. The test by analysis of variance shows that there was no significant difference between the line means, although there was a significant difference between successive generations. In strains II, III, and IV there were no significant differences between either the line means or the number of progeny produced in successive parthenogenetic generations.

TABLE 2

SHOWING TEST FOR STRAIN HOMOGENEITY IN STRAIN II

	Serial Number of Strain									
	1	2	3	4	5	6	7	8	9	10
Av.....	39.4	38.8	40	40.2	41.8	41.4	40.8	38.2	37.8	36.6

Mean, 39.5±0.50. Standard deviation, 1.58±0.35. Range from 36.6±to 41.8
Test by analysis of variance shows no significance

TABLE 3

SHOWING TEST FOR STRAIN HOMOGENEITY IN STRAIN III

	Serial Number of Strain									
	1	2	3	4	5	6	7	8	9	10
Av.....	48.6	46.2	50.4	49.6	48.4	52.6	50	49.2	51.4	47

Mean, 49.34±0.58. Standard deviation, 1.81±0.41. Range from 46.2 to 52.6
Test by analysis of variance shows no significance

The parental stock of strain II had an average number of progeny of 39.5 ± 0.50. The standard deviation was 1.58 ± 0.35, and the range was from 36.6 to 41.8 (Table 2). In strain III the mean number of progeny produced was 49.34 ± 0.58, with a standard deviation of 1.81 ± 0.41 and a range of from 46.2 to 52.6 (Table 3). Strain IV had an

[4] Biometric constants were determined in order to compare the results of experiments in this particular investigation. The writer realizes that the number of individuals involved in this investigation are not sufficient accurately to apply the normal probability curve.

average number of progeny of 51.74 ± 0.62 in five parthenogenetic generations (Table 4). The standard deviation was 1.96 ± 0.44, and the range was from 48 to 54.

A study was made of the number of progeny produced by several members of a parthenogenetic family in order to determine whether the members of one family displayed the same degree of vigor. The first twenty and the last fifteen members of its

TABLE 4

SHOWING TEST FOR STRAIN HOMOGENEITY IN STRAIN IV

	Serial Number of Strain									
	1	2	3	4	5	6	7	8	9	10
Av.....	50	49	48	52.4	51.6	52.4	53	53.2	54	53.8

Mean, 51.74±0.62. Standard deviation, 1.96±0.44. Range from 48 to 54
Test by analysis of variance shows no significance

TABLE 5

SHOWING NUMBER OF YOUNG FEMALES PRODUCED BY THIRTY-FIVE LINES ALL OF WHICH STARTED FROM ONE PARTHENOGENETIC FAMILY

The first females of lines 1–20 were the first members of the family to hatch; the first females of lines 21–35 were the last members of the family to hatch.

Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.
1.....	49.4	10.....	45	19.....	47	28.....	42.8
2.....	50	11.....	45.6	20.....	48	29.....	45.8
3.....	50.2	12.....	45.2	21.....	48.8	30.....	43.8
4.....	47.8	13.....	50	22.....	43.2	31.....	49.6
5.....	48	14.....	43.6	23.....	45.4	32.....	43.4
6.....	51.6	15.....	49.2	24.....	47	33.....	44.4
7.....	50	16.....	46.4	25.....	48	34.....	43.4
8.....	45.4	17.....	45.8	26.....	49.4	35.....	46.8
9.....	49.4	18.....	49.2	27.....	48		

Mean, 47.03±0.41. Standard deviation, 2.44±2.92. Range from 42.8 to 51.6
Test by analysis of variance shows significance between line means
but no significance between generations

parthenogenetic family were each isolated in a watch glass, and the vigor of each line was determined. The results of this study (Table 5) show that the mean number of progeny produced was 47.03 ± 0.41, the standard deviation was 2.44 ± 2.92, and the range was from 42.8 to 51.6. The test by analysis of variance shows a significance between line means. Theoretically, there should be no significant difference, since all the lines were derived from the same mother through parthenogenetic reproduction. As may be noted from the standard deviation, there was no great variation in the degree of vigor expressed by the thirty-five lines. The deviation which did occur, as is shown in

the test by analysis of variance, might have been due to experimental error, such as slight changes in environmental conditions over the period of time in which the vigor was being determined, or to changes in the genetic constitution of the rotifers in some of the lines.

THE EFFECTS OF INBREEDING

It has been demonstrated that in parthenogenetic reproduction in *Hydatina* the members of one family are relatively uniform in vigor. This condition is theoretically correct, inasmuch as in *Hydatina* the parthenogenetic egg has only one maturation division, without reduction, and remains diploid, so that the complement of chromosomes with their genes is presumably identical throughout parthenogenetic reproduction.

The offspring derived from bisexual reproduction within a family often vary profoundly in the degree of vigor expressed. This condition also agrees with the theoretical expectations. In bisexual reproduction, two haploid gametes, ordinarily genetically unlike, produce the zygote. The number of variations which occur in bisexual reproduction

TABLE 6

THE S_1 GENERATION OF STRAIN I: SHOWING THE NUMBER OF YOUNG FEMALES PRODUCED BY TWENTY LINES, EACH FROM A FERTILIZED EGG

Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.
1.....	43.8	6.....	51.6	11.....	32.2	16.....	16.2
2.....	54.2	7.....	48.2	12.....	53.4	17.....	51
3.....	32.8	8.....	10.8	13.....	56.2	18.....	58.2
4.....	7.4	9.....	45.8	14.....	43	19.....	53.2
5.....	13.6	10.....	52.4	15.....	9	20.....	54.8

Mean, 39.34 ± 3.94. Standard deviation, 17.60 ± 2.78. Range from 7.2 to 58.2

depends on the number of chromosomes and their contained genes, as well as on the degree of heterozygosity of the parental stock. Bisexually produced offspring usually show, by the number of variations in their degree of vigor, that the individual from which they have arisen is, to some degree, heterozygous.

Each bisexually produced individual should give rise parthenogenetically to a family whose genetic makeup is the same as that of the originally hatched individual. Experimental verifications of these expectations were shown in the discussion on the effects of parthenogenetic reproduction.

An analysis and discussion of the effects of bisexual inbreeding on each of the four strains of rotifers used in this investigation follows. Strain I, with an average index of vigor of 41.14 ± 0.33 (Table 1), was inbred, and twenty eggs were hatched (Table 6). The mean number of progeny produced by these twenty individuals in five generations became 39.34 ± 3.94, the standard deviation 17.60 ± 2.78, and the range was from 7.2 to 58.2. The standard deviation and range are especially significant in analyzing these data, since they indicate the degree of variation which has taken place. Deviation results from segregation into lines having different degrees of vigor.

Line No. 4 of the S_1 generation, with an index of vigor of 7.2, was inbred. The results show that this strain was heterozygous for the factors involved in vigor, as was the

parental stock. Thirty eggs were hatched in this generation. The mean number of progeny produced was 29.36 ± 2.07, the standard deviation was 11.31 ± 1.46, and the range was from 3.6 to 44.2 (Table 7).

In third generation, line No. 5 of the second generation was inbred. Line No. 5 had an index of vigor of 3.6 (Table 8). The results show that segregation into lines with different

TABLE 7

THE S_2 GENERATION OF STRAIN I: SHOWING THE NUMBER OF YOUNG FEMALES PRODUCED BY THIRTY LINES, EACH FROM A FERTILIZED EGG LAID BY INBRED FEMALES OF LINE NO. 4 OF THE S_1 GENERATION

Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.
1	28.2	9	20.2	17	26.6	25	38.6
2	42.8	10	25.6	18	44.2	26	41.2
3	35.8	11	20.8	19	41.2	27	28.6
4	40.6	12	38.8	20	33.8	28	23
5	3.6	13	36	21	28	29	39.8
6	24	14	12.2	22	38.8	30	7.2
7	35.6	15	3.8	23	34.8		
8	27.4	16	21.6	24	37.6		

Mean, 29.36 ± 2.07. Standard deviation, 11.31 ± 1.46. Range from 3.6 to 44.2

TABLE 8

THE S_3 GENERATION OF STRAIN I: SHOWING THE NUMBER OF YOUNG FEMALES PRODUCED BY THIRTY LINES, EACH FROM A FERTILIZED EGG LAID BY INBRED FEMALES OF LINE NO. 5 OF THE S_2 GENERATION

Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.
1	5	9	28.6	17	27	25	33.2
2	25.4	10	32.6	18	50.8	26	44.6
3	7.4	11	11.4	19	15	27	32.2
4	28.4	12	6.2	20	35.8	28	22.6
5	29.8	13	31.2	21	38	29	34.8
6	26.2	14	43	22	50	30	43
7	18.4	15	41.8	23	36.4		
8	36.6	16	23.2	24	31		

Mean, 29.68 ± 2.22. Standard deviation, 12.16 ± 1.57. Range from 5 to 50.8

degrees of vigor had occurred. The average number of progeny prouced was 29.68 ± 2.22, the standard deviation was 12.16 ± 1.57, and the range was from 5 to 50.8.

The effects of inbreeding in strain I are not so pronounced, so far as reaching a homozygous condition is concerned, as they were in the other three strains. The average number of progeny produced by the strain dropped from 44.14 ± 0.46 in the parental stock to 29.68 ± 2.22 in the third generation of inbreeding. This decline indicates a definite weakening of the strain; but a general degree of weakness was not evidenced for

all individuals, as is shown by the range, which was from 5 to 50.8 in the third generation. There was a slight gain in the average number of progeny produced in the third generation over that produced in the second generation. This condition is true only for strain I of the four strains investigated. Further inbreeding of strain I might have decreased its vigor even more.

The effects of inbreeding in strain II are more striking than in strain I. The parental stock had an average index of vigor of 39.5 ± 0.501 (Table 2). The parental was also

TABLE 9

THE S_1 GENERATION OF STRAIN II: SHOWING THE NUMBER OF YOUNG FEMALES PRODUCED BY TWENTY LINES, EACH FROM A FERTILIZED EGG

Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.
1	12.6	6	51.2	11	17.2	16	53.2
2	46	7	33.8	12	53.6	17	50.2
3	47.4	8	13.2	13	50	18	39.8
4	48	9	6.4	14	51.6	19	16.4
5	49	10	36.4	15	52.6	20	34.2

Mean, 38.14±3.51. Standard deviation, 15.79±2.48. Range from 6.4 to 53.6

TABLE 10

THE S_2 GENERATION OF STRAIN II: SHOWING THE NUMBER OF YOUNG FEMALES PRODUCED BY THIRTY LINES, EACH FROM A FERTILIZED EGG LAID BY INBRED FEMALES OF LINE NO. 1 OF THE S_1 GENERATION

Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.
1	8.2	9	10.4	17	6.6	25	15.8
2	11.8	10	7	18	6.2	26	10.8
3	5.2	11	3.4	19	9.6	27	17.4
4	12.6	12	12.2	20	5	28	3.4
5	9.2	13	4.4	21	7.8	29	9.6
6	2.8	14	13.8	22	12	30	17.8
7	3	15	8	23	7.8		
8	7.2	16	6	24	6.8		

Mean, 8.7±0.74. Standard deviation, 4.04±0.52. Range from 2.8 to 17.8

inbred bisexually. Twenty eggs were hatched, and the progeny of the females raised through five parthenogenetic generations. The results of this inbreeding show that the mean was lowered to 38.14 ± 3.51 (Table 9). The standard deviation became 15.79 ± 2.48, and the range was from 6.4 to 53.6. These figures indicate the amount of variation, so far as vigor is concerned, which resulted from this first inbreeding.

Line No. 1, with an index of vigor of 12.6, was inbred to the S_2 generation. In the second generation the mean number of progeny produced by thirty individuals in five parthenogenetic generations was 8.7 ± 0.74, the standard deviation was 4.04 ± 0.52, and the range was from 2.8 to 17.8 (Table 10).

Line No. 6 of the second generation, whose index of vigor was 2.8, was inbred. Thirty individuals were hatched from fertilized eggs. In the S_3 generation the mean number of young females produced was 8.38 ± 1.50, the standard deviation from the mean was 8.35 ± 1.08, and the range was from 1.2 to 36.6 (Table 11).

The effects of inbreeding on strain III are similar to those of strain II. The parental stock had an average number of progeny of 49.34 ± 0.58 (Table 3). This stock was inbred, twenty eggs were hatched, and the progeny was recorded for five generations.

TABLE 11

THE S_3 GENERATION OF STRAIN II: SHOWING THE NUMBER OF YOUNG FEMALES PRODUCED BY THIRTY LINES, EACH FROM A FERTILIZED EGG LAID BY INBRED FEMALES FROM LINE NO. 6 OF THE S_2 GENERATION

Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.
1	12.8	9	2.2	17	3	25	5.6
2	2.2	10	9	18	12.4	26	5.6
3	8.6	11	15	19	3.2	27	4.2
4	7.4	12	2.4	20	33.4	28	4.6
5	3	13	7.2	21	4	29	4.8
6	8.2	14	6.2	22	1.2	30	35.6
7	6	15	21.2	23	3		
8	4.8	16	11.2	24	2.4		

Mean, 8.38±1.50. Standard deviation, 8.35±1.08. Range from 1.2 to 36.6

TABLE 12

THE S_1 GENERATION OF STRAIN III: SHOWING THE NUMBER OF YOUNG FEMALES PRODUCED BY TWENTY LINES, EACH FROM A FERTILIZED EGG

Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.
1	49	6	43.8	11	19	16	32.8
2	51	7	54.8	12	44.2	17	42.8
3	14.4	8	46	13	43.6	18	37.4
4	48.2	9	48.4	14	44	19	32
5	50.6	10	49	15	13	20	35.6

Mean, 40.00±2.66. Standard deviation, 11.88±1.88. Range from 13 to 54.8

The mean became 40.00 ± 2.66, the standard deviation 11.88 ± 1.88, and the range was from 13 to 54.8 (Table 12).

Line No. 15 of the S_1 generation, with an index of 13, was then inbred. In the progeny of thirty individuals, which were raised through five parthenogenetic generations, the mean became 21.23 ± 1.73, the standard deviation 9.50 ± 1.23, and the range was from 8.8 to 42.8 (Table 13).

In the third generation, line No. 15 of the S_2 generation, with an index of vigor of 12.2, was inbred. Thirty individuals were again hatched, and the index of vigor of the

thirty lines was determined. The results obtained were as follows: mean, 12.9 ± 0.92; standard deviation, 5.02 ± 0.65; and the range, from 3.2 to 21.8 (Table 14).

The parental stock of strain IV had an average number of progeny of 51.74 ± 0.62 (Table 4). In the S_1 generation the mean became 44.24 ± 2.21 for twenty individuals lines (Table 15). The standard deviation for the twenty lines was 9.88 ± 1.56, and the

TABLE 13

THE S_2 GENERATION OF STRAIN III: SHOWING THE NUMBER OF YOUNG FEMALES PRODUCED BY THIRTY LINES, EACH FROM A FERTILIZED EGG LAID BY INBRED FEMALES OF LINE NO. 15 OF THE S_1 GENERATION

Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.
1.....	15.2	9.....	26	17.....	29.8	25.....	13.8
2.....	42.2	10.....	11.8	18.....	13.8	26.....	35.4
3.....	8.8	11.....	14.6	19.....	12.2	27.....	22
4.....	26.2	12.....	10.6	20.....	18.6	28.....	14.8
5.....	32.6	13.....	22.8	21.....	18.4	29.....	23.4
6.....	17.8	14.....	36.4	22.....	15.4	30.....	9.6
7.....	42.8	15.....	12.2	23.....	15.6		
8.....	29.4	16.....	28	24.....	16.6		

Mean, 21.23 ± 1.73. Standard deviation, 9.50 ± 1.23. Range from 8.8 to 42.8

TABLE 14

THE S_3 GENERATION OF STRAIN III: SHOWING THE NUMBER OF YOUNG FEMALES PRODUCED BY THIRTY LINES, EACH FROM A FERTILIZED EGG LAID BY INBRED FEMALES OF LINE NO. 15 OF THE S_2 GENERATION

Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.
1.....	5.8	9.....	10.6	17.....	8.4	25.....	20.4
2.....	3.2	10.....	11.8	18.....	9.2	26.....	11.4
3.....	16.4	11.....	14.4	19.....	5.8	27.....	12.8
4.....	16.8	12.....	10.8	20.....	15.4	28.....	19.8
5.....	12	13.....	21.8	21.....	17.8	29.....	8.4
6.....	10.8	14.....	12.6	22.....	18.4	30.....	3.8
7.....	16.4	15.....	14.6	23.....	18.8		
8.....	9	16.....	9.6	24.....	20.4		

Mean, 12.9 ± 0.92. Standard deviation, 5.02 ± 0.65. Range from 3.2 to 21.8

range was from 13.6 to 54.8. For the S_2 generation, line No. 12 from the S_1 generation, with an index of vigor of 13.6, was inbred. Thirty individuals were hatched, and the index of vigor for the respective lines was determined. The mean number of young females produced was 27.48 ± 1.05; the standard deviation from the mean was 11.23 ± 1.45; and the range was from 7 to 46.4 (Table 16).

Line No. 18 from the S_2 generation, with an expression of vigor of 9.8 young per parthenogenetic generation, was inbred. In thirty lines which were raised, the mean

number of progeny produced was 11.08 ± 0.85; the standard deviation, 4.65 ± 0.60; and the range, from 5.1 to 21.2 (Table 17).

The results of inbreeding in strain IV are more typical for the four strains of *Hydatina* used in this study. Starting with the parental stock, the individuals were heterozygous, as is indicated by the results of inbreeding to the first generation. Some of the individuals in the first inbred generation were as strong as the parental stock, while other were much weaker. Vigor, in general, decreased considerably.

TABLE 15

THE S_1 GENERATION OF STRAIN IV: SHOWING THE NUMBER OF YOUNG FEMALES PRODUCED BY TWENTY LINES, EACH FROM A FERTILIZED EGG

Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.
1.....	52.2	6.....	45.2	11.....	33.6	16.....	50.2
2.....	51.2	7.....	54.8	12.....	13.6	17.....	44.8
3.....	43.2	8.....	49.8	13.....	52.4	18.....	26
4.....	45.8	9.....	47	14.....	42.6	19.....	49.4
5.....	47.2	10.....	49.6	15.....	35	20.....	51.2

Mean, 44.24 ± 2.21. Standard deviation, 9.88 ± 1.56. Range from 13.6 to 54.8

TABLE 16

THE S_2 GENERATION OF STRAIN IV: SHOWING THE NUMBER OF YOUNG FEMALES PRODUCED BY THIRTY LINES, EACH FROM A FERTILIZED EGG LAID BY INBRED FEMALES OF LINE NO. 12 OF THE S_1 GENERATION

Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.
1.....	30.4	39.....	44.4	17.....	34.8	25.....	26.4
2.....	19	10.....	44	18.....	9.8	26.....	15
3.....	17.2	11.....	19	19.....	22.4	27.....	7
4.....	17.4	12.....	23.8	20.....	14.8	28.....	20
5.....	18.8	13.....	42	21.....	22.8	29.....	21.8
6.....	46.4	14.....	43.4	22.....	31.4	30.....	21
7.....	30.4	15.....	42	23.....	23.8		
8.....	35.6	16.....	42.4	24.....	28.4		

Mean, 27.48 ± 1.05. Standard deviation, 11.23 ± 1.45. Range from 7 to 46.4

The results of inbreeding and selection to the second generation are more interesting to analyze. In this generation some of the lines greatly surpassed the vigor of the line from the first generation, whose parthenogenetic progeny was inbred. In inbreeding this line, as well as all others, the male and female, although they were both parthenogenetic progeny of the same individual, need not have the same genetic makeup. In the reduction division to the haploid condition in males and mictic females, a random assortment of the chromosomes may take place which will alter the genetic makeup of the resulting progeny. If the parents were homozygous for the factors involved in vigor, both the male

and the female progeny of the individual in parthenogenetic reproduction would have the same genetic makeup. If the parents were heterozygous for the factors involved, which is apparently the case in all the strains studied in this investigation, the male and female would carry different factors for the determination of vigor. In inbreeding strain IV to the S_2 generation it was found that, out of thirty individual lines which were studied, two were weaker than the parent, while twenty-eight were stronger. Because of experimental error, one could hardly expect that the inbred progeny, either bisexual or parthenogenetic, would ever show exactly the same degree of vigor as the parent. In instances of bisexual inbreeding, however, there were great extremes of increased vigor, as well as some loss in vigor. In the S_2 generation line No. 6 had an index of vigor of 46.4; No. 9, 44.4; and No. 10, 44; while No. 27 had an average of only seven young females for each of the five parthenogenetic generations (Table 12).

TABLE 17

THE S_3 GENERATION OF STRAIN IV: SHOWING THE NUMBER OF YOUNG FEMALES PRODUCED BY THIRTY LINES, EACH FROM A FERTILIZED EGG LAID BY INBRED FEMALES OF LINE NO. 18 OF THE S_2 GENERATION

Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.
1.....	13	9.....	6.2	17.....	8.4	25.....	6
2.....	7	10.....	16.2	18.....	5.6	26.....	17
3.....	7.6	11.....	7.6	19.....	10	27.....	9.4
4.....	16	12.....	12.8	20.....	10.8	28.....	7
5.....	11.4	13.....	12	21.....	9.8	29.....	20.6
6.....	17.4	14.....	5.8	22.....	11	30.....	12.4
7.....	12	15.....	5.1	23.....	19.2		
8.....	8.4	16.....	5.4	24.....	21.2		

Mean, 11.08 ± 0.85. Standard deviation, 4.65 ± 0.6. Range from 5.1 to 21.2

In the S_3 generation of strain IV there occurred results similar to those described for the S_2 generation. Line No. 18 of the S_2 generation, with an index of vigor of 9.9, was inbred. Of thirty lines which were raised from this inbreeding, thirteen were weaker than the parental stock of the S_2 generation, while seventeen were as strong or stronger. The range from the weakest to the strongest line was from 5.1 to 21.2 (Table 17). This range indicates that there was a more homozygous condition in the S_3 generation than in the preceding ones. The standard deviation of 4.65 ± 0.60 also indicates that the S_3 generation, in comparison to the S_1 and S_2 generations, was relatively homozygous.

In reviewing the data which were obtained from the above studies on inbreeding, two questions may arise, namely, "Was the size of the parthenogenetic family a measure of vigor expressed as a single character which is possibly controlled by multiple factors?" and "In inbreeding the weak lines, why do most of the progeny exceed the parent in the size of the parthenogenetic family?"

Concerning the first question, the writer believes that the size of the parthenogenetic family is a measure of vigor and that it is controlled by multiple factors. The behavior of the individual rotifers making up the weak lines, in contrast to those of strong lines, indicates the same. In the weak lines the adult females did not display vigorous move-

ments and locomotion, as did the famales of the strong lines. The rotifers of the weak lines seemed to be subject to a congestion of bacteria and debris in the digestive tract, resulting in inactivity and death. Difficulty in starting successive parthenogenetic generations was also encountered in culturing the weak lines. Size measurements were not made; but, as judged from careful observations of hundreds of individuals from both strong and weak lines, the individuals from weak lines seemed to be somewhat smaller. After working for several months with both strong and weak lines of *Hydatina* one can usually separate the weak from the strong lines by general observation of the individuals, without counting the number of young females produced.

As has been noted, a small percentage of the fertilized eggs hatched in both weak and strong lines. It is possible that in inbreeding weak lines many of the eggs which may have yielded weak individuals did not hatch. This condition might account for the fact that many of the progeny of weak inbred lines were more vigorous than the parent. The results obtained in these studies are unusual in this respect. Although no actual data are on hand to substantiate the above proposal, it was observed that weak individuals had a more difficult time in hatching, as was evidenced by the usually increased length of time required for hatching.

From the data which are on hand from the studies on inbreeding, it seems as though multiple factors are involved in the determination of vigor in *Hydatina*. Considerable variation occurs upon inbreeding any of the lines. Each line breeds relatively true parthenogenetically. Because of the small number of rotifers which were raised in this investigation, it would be quite impossible to determine the number of factors involved in the determination of the quantitative expression of vigor. The cause of the variations which exist among strains after inbreeding is, of course, due to the random assortment of chromosomes which takes place in the production of haploid males and mictic females. During fertilization it is the chance meeting of the various factors controlling vigor which determines the degree of vigor expressed in the progeny.

Banta (1936) in a summary of his extensive investigations in inheritance with *Daphnia longispina*, which reproduces similarly to *H. senta*, suggests that recessive mutations accumulate during parthenogenesis and make their presence known in bisexual inbreeding within a clone. The effects revealed by these mutant factors are similar to the effects which resulted from inbreeding *H. senta*—especially the effect of a wide range of vegetative vigor. The writer has taken these possibilities into consideration in analyzing the effects of inbreeding on *Hydatina*. Recessive mutations may certainly arise during parthenogenetic reproduction in *Hydatina*, but from the data which have been collected from the results of bisexual inbreeding the idea of multiple factors which control vigor seems very plausible. In the number of rotifers which were raised in this investigation, recessive mutations would probably not occur in sufficient numbers to bring about the great variations in degrees of vigor which have resulted from bisexual inbreeding.

Theoretically, it should be possible to obtain pure strains of strong and weak rotifers. In this investigation the closest approach to a pure homozygous condition was that of the third generation of inbreeding strain IV (Table 17). In this generation the strongest line had an index of vigor of 21.2; and the weakest, 5.1. Although there is still considerable variation, this generation is the closest approach to a homozygous condition of weakness which was encountered in the present investigation. In comparison with the vigor of wild cultures of *Hydatina* (Tables 3 and 4), this is a relatively weak homozygous race.

THE EFFECTS OF CROSSBREEDING

It has been shown in the preceding description of experiments in this investigation that in inbreeding strains of *Hydatina* vigor is greatly reduced. The effects of crossbreeding upon the same strains that were inbred will now be discussed.

At the beginning of the investigation strains I and II were crossed reciprocally. The eggs were hatched from each cross, and the vigor of the resulting lines was determined. In the cross between strain I (♂) and strain II (♀) the mean number or progeny produced was 37.20 ± 2.76, the standard deviation was 8.72 ± 1.95, and the range was

TABLE 18

CROSS BETWEEN THE PARENTAL STOCK OF STRAIN I (♂) AND STRAIN II (♀) SHOWING THE NUMBER OF YOUNG FEMALES PRODUCED BY TEN LINES EACH FROM A FERTILIZED EGG OF THE ABOVE CROSS

Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.
1.....	40.8	4.....	37	7.....	21.8	10.....	37.8
2.....	46.6	5.....	45	8.....	25.8		
3.....	27.8	6.....	40.8	9.....	48.6		

Mean, 37.20 ± 2.76. Standard deviation, 8.72 ± 1.95. Range from 27.8 to 48.6

TABLE 19

CROSS BETWEEN THE PARENTAL STOCK OF STRAIN I (♀) AND STRAIN II (♂) SHOWING THE NUMBER OF YOUNG FEMALES PRODUCED BY TEN LINES EACH FROM A FERTILIZED EGG OF THE ABOVE CROSS

Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.
1.....	35.2	4.....	47	7.....	31.8	10.....	38.8
2.....	46.2	5.....	35.4	8.....	19.2		
3.....	29.2	6.....	30	9.....	24.4		

Mean, 33.72 ± 2.64. Standard deviation, 8.36 ± 1.87. Range from 19.2 to 47

from 27.8 to 48.6 (Table 18). In the reciprocal cross between strain I (♀) and strain II (♂) the mean became 33.72 ± 2.64, the standard deviation was 8.36 ± 1.87, and the range was from 19.2 to 46.2 (Table 19). These data indicate that variation resulted from crossbreeding, but not the to same extent that it occurred in most of the inbred generations. Some lines, such as No. 2 of the strain I (♀) and II (♂) cross, were stronger than the parental stock (Tables 1 and 2); and other lines, such as No. 6 of the I (♀) and II (♂) cross, were weaker. Such variations may be expected to result invariably from a cross between heterozygous strains.

Strain III, with an average number of progeny of 49.34 ± 0.58 in its original condition, was crossed with strain IV, which had an index of vigor of 51.74 ± 1.62 in its original state. Table 20 gives the results of this cross. The mean number of progeny

for thirty individual lines became 45.32 ± 1.75, the standard deviation was 9.60 ± 1.24, and the range was from 25 to 55.6. These results were similar to those obtained from the first cross between strains I and II. No reciprocal cross was made between strains III and IV. The amount of variation which occurred in these crosses, as is indicated by the range and standard deviation, further verifies the idea that the strains were heterozygous at the beginning of the investigation. Multiple factors controlling the extent of vigor are probably present, as has been discussed previously. The presence of multiple factors is evidenced by the fact that various degrees of vigor, depending upon the number of factors present, are represented in the progenies of the crosses between the strains. No complete dominance or recessiveness is indicated.

After the four strains were inbred for three generations, a weak line from each strain was crossed with a line from another, in the same manner as has been described for the

TABLE 20

CROSS BETWEEN THE PARENTAL STOCK OF STRAIN III (♂) AND STRAIN IV (♀) SHOWING THE NUMBER OF YOUNG FEMALES PRODUCED BY THIRTY LINES EACH FROM A FERTILIZED EGG OF THE ABOVE CROSS

Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.	Serial No. of Strain	Av.
1.....	31	9.....	49.6	17.....	55.6	25.....	40.2
2.....	27.8	10.....	52	18.....	52.6	26.....	46.8
3.....	42.6	11.....	52	19.....	52.6	27.....	49.2
4.....	25	12.....	40.4	20.....	53.8	28.....	54.8
5.....	28.4	13.....	52.4	21.....	54.8	29.....	46.8
6.....	28	14.....	55.2	22.....	48	30.....	53.6
7.....	31.8	15.....	51.4	23.....	45.8		
8.....	36.6	16.....	49.4	24.....	52.4		

Mean, 45.32±1.75. Standard deviation, 9.60±1.24. Range from 25 to 55.8

crossing of the lines in the original stock. Twenty-five eggs were hatched from each cross, and a reciprocal cross was not made.

In the first single cross, line No. 3 of strain I, with an index of vigor of 7.4, was crossed with line No. 1 of strain III, which had an index of vigor of 5.8. The results of this hybridization are given in Table 21. The average number of progeny produced by twenty-five lines was 23.15 ± 4.87, the standard deviation was 24.34 ± 48, and the range was from 11.4 to 49.8. There was an increase of 213 per cent over line No. 3 of strain I and an increase of 299 per cent over line No. 1 from strain III.

Line No. 10 of the S_3 generation of strain II was crossed with line No. 14 of the S_3 generation of strain IV. Line No. 10 from strain II had an index of vigor of 9.0, and line No. 14 from strain IV had an index of vigor of 5.8. Upon crossing these two lines (Table 22) the average number of progeny produced by twenty-five lines was 21.32 ± 4.63, the standard deviation was 23.15 ± 3.30, and the range was from 2.2 to 40.0. The average rate of reproduction of 21.28 represents an increase of 125 per cent over the rate for line No. 10 from strain II and of 267 per cent over line No. 14 from strain IV.

The increase in vigor which was displayed in the single crosses corresponds to what occurs in similar conditions in other organisms. Several theories have been advanced by

various investigators concerning the cause of hybrid vigor. In *Hydatina*, hybrid vigor seems to be due to the presence of numerous genes or multiple factors which control the vigor of the organism, as is displayed in the rate of reproduction. These genes are probably located on different chromosomes. Vigor is then determined by the number of dominant genes which are present in the cells of the rotifer. When the recessive genes are brought together in a homozygous condition, the strain is weakened. Crosses between inbred lines may bring more of the dominant genes together in the hybrid, provided

TABLE 21

CROSS BETWEEN LINE NO. 3 OF THE S_3 GENERATION OF STRAIN I AND LINE NO. 1 OF THE S_3 GENERATION OF STRAIN III

Serial No. of Line	Av.	Serial No. of Line	Av.	Serial No. of Line	Av.	Serial No. of Line	Av.
1.....	19.6	8.....	17.4	15.....	22.0	22.....	22
2.....	20	9.....	25.0	16.....	15	23.....	18.6
3.....	21.0	10.....	26.8	17.....	16	24.....	23.2
4.....	11.4	11.....	28.4	18.....	17.6	25.....	15.2
5.....	34.4	12.....	49.8	19.....	23.6		
6.....	27.0	13.....	26.0	20.....	18.6		
7.....	25.4	14.....	30.2	21.....	24.6		

Mean, 23.15 ± 4.87. Standard deviation, 24.34 ± 3.48. Range from 11.4 to 49.8

TABLE 22

CROSS BETWEEN LINE NO. 10 OF THE S_3 GENERATION OF STRAIN II AND LINE NO. 14 OF THE S_3 GENERATION OF STRAIN IV

Serial No. of Line	Av.	Serial No. of Line	Av.	Serial No. of Line	Av.	Serial No. of Line	Av.
1.....	35.0	8.....	40.0	15.....	26.8	22.....	30.6
2.....	18.4	9.....	2.4	16.....	20.2	23.....	19.6
3.....	17.4	10.....	17.4	17.....	35.0	24.....	18.2
4.....	17.0	11.....	2.2	18.....	15.6	25.....	17.4
5.....	20.6	12.....	21.2	19.....	15.8		
6.....	20.4	13.....	33.8	20.....	26.2		
7.....	8.2	14.....	27.4	21.....	26.2		

Mean, 21.32 ± 4.63. Standard deviation, 23.15 ± 3.30. Range from 2.2 to 40.0

each inbred line carries the vigor factors which the other lacks. All crosses between inbred lines do not result in the same degree of heterosis.

From the results of hybridization of inbred lines in other organisms, such as corn, one might expect the progeny of the single crosses in *Hydatina* to equal the parental stocks in the degree of vigor they express. Some lines did equal the strength of the parental stocks; others approached it closely; while many were quite weak. The weakness of lines after crossing may be due to the loss of some dominant factors for vigor in the inbreeding of the weakest line in each generation of inbreeding. Further investigations in the inbreeding of the strongest lines of each generation will probably give valuable information of this point.

TABLE 23

A SUMMARY OF THE BIOMETRIC CONSTANTS DETERMINED FROM THE NUMBER OF YOUNG FEMALES PRODUCED BY THE PARENTAL STOCKS, THE BISEXUALLY INBRED LINES, AND THE BISEXUALLY CROSSBRED LINES OF THE FOUR STRAINS OF ROTIFERS USED IN THIS INVESTIGATION ON THE PHENOMENON OF VIGOR (METHOD OF SELECTION ALSO GIVEN)

Strain	Method of Selection	Mean	Standard Deviation
	Strain I		
Parthenogenetic parental stock	One individual at random	41.14±0.46	1.47±0.33
Bisexual inbreeding:			
S_1 generation	Parental stock (41.14)*	39.34±3.94	17.60±2.78
S_2 generation	Line No. 4 of S_1 (7.2)	29.36±2.07	11.31±1.46
S_3 generation	Line No. 5 of S_2 (3.6)	29.68±2.22	12.16±1.57
	Strain II		
Parthenogenetic parental stock	One individual at random	39.50±0.50	1.58±0.35
Bisexual inbreeding:			
S_1 generation	Parental stock (39.50)	38.14±3.51	15.70±2.48
S_2 generation	Line No. 1 of S_1 (12.6)	8.70±0.74	4.04±0.52
S_3 generation	Line No. 6 of S_2 (2.8)	8.40±1.50	8.36±1.08
	Strain III		
Parthenogenetic parental stock	One individual at random	49.34±0.58	1.81±0.41
Bisexual inbreeding:			
S_1 generation	Parental stock (49.34)	40.00±2.65	11.88±1.87
S_2 generation	Line No. 15 of S_1 (13.0)	21.23±1.73	9.50±1.22
S_3 generation	Line No. 15 of S_2 (12.2)	12.90±0.92	5.02±0.65
	Strain IV		
Parthenogenetic parental stock	One individual at random	51.74±0.62	1.96±0.44
Bisexual inbreeding:			
S_1 generation	Parental stock (51.74)	44.24±0.22	9.88±1.56
S_2 generation	Line No. 12 of S_1 (13.6)	27.48±1.05	11.23±1.45
S_3 generation	Line No. 18 of S_2 (9.8)	11.08±0.95	4.65±0.60
	Cross between Parental Stocks		
Strain I (♂) and strain II (♀)	Parental stocks (41.14) (39.50)	37.20±2.76	8.72±1.95
Strain I (♀) and strain II (♂)	Parental stocks (39.50) (41.14)	33.72±2.64	8.36±1.87
Strain III (♂) and strain IV (♀)	Parental stocks (49.34) (51.74)	45.32±1.75	9.60±1.24
	Single Crosses after Three Generations of Inbreeding		
Strain I (♂) and strain III (♀)	Line of No. 3 of S_3 (7.4) and line No. 1 of S_3 (5.8)	23.15±4.87	24.34±3.48
Strain II (♂) and strain IV (♀)	Line No. 10 of S_3 (9.0) and line No. 14 of S_3 (5.8)	21.32±4.63	23.15±3.30

* Numbers in parentheses indicate vigor of line before inbreeding and crossing.

More than four thousand female rotifers, which produced over ninety-four thousand young, were raised and recorded during this investigation. This number includes those that were raised in the preliminary work of perfecting the technique in culturing the rotifers. The number of male rotifers raised was not recorded.

SUMMARY

1. Buffer solutions were used to maintain a pH of 7.2 in the culture solutions at a temperature of from 16° to 18° C. The flagellate *Polytoma* was added daily as food.

2. The hatching of fertilized eggs was made possible by placing them for 3 days in a desiccator containing CaCl crystals.

3. The rate of parthenogenetic reproduction within a given line was found to be uniform.

4. Bisexual inbreeding at first resulted in a variety of lines with different degrees of vigor, ranging from seven daughters by a mother in the lowest to fifty-eight daughters by a mother in the highest in any one generation.

5. Weak homozygous lines were obtained by selection and continual inbreeding for three generations.

6. Crossbreeding and selection after three generations of inbreeding restored vigor to a marked degree.

7. Multiple factors are probably responsible for the variation in vigor in *Hydatina*.

8. Over four thousand female rotifers, which produced more than ninety-four thousand young, were raised and recorded during this investigation.

9. Table 23 gives a summary of the biometric constants which were determined from these studies.

LITERATURE CITED

BANTA, A. M. 1936. Preparation of report on *Cladocera* studies. Yearbook Carnegie Inst. Wash., No. 36, pp. 309–12.

EAST, E. M., and HAYES, H. K. 1912. Heterzygosis in evolution and in plant breeding. U.S. Bur. Plant Indus., No. 243. Pp. 58.

EAST, E. M., and JONES, D. F. 1919. Inbreeding and outbreeding. Philadelphia: J. B. Lippincott Co. Pp. 285.

FERRIS, J. C. 1932. A comparison of the life histories of mictic and amictic females in the rotifer, *Hydatina senta*. Biol. Bull., 63:442–55.

MATHEWS, A. P. 1931. Physiological chemistry. New York: William Wood & Co. Pp. 1154.

SHULL, A. F. 1912. The influence of food in controlling sex in *Hydatina senta*. Biol. Bull., 24:1–13.

SNEDECOR, GEORGE W. 1940. Statistical methods. Ames: Iowa State College Press. Pp. 422.

WHITNEY, D. D. 1907. Determination of sex in *Hydatina senta*. Jour. Exper. Zoöl., 5:1–26.

———. 1914. The influence of food in controlling sex in *Hydatina senta*. *Ibid.*, 17:545–58.

STUDIES ON THE NUTRITIONAL REQUIREMENTS OF EUGLENA GRACILIS IN DARKNESS

HENRY W. SCHOENBORN
Biological Laboratory, University College, New York University, and
Department of Zoölogy, University of Oregon

A COMPARISON of the nutritional requirements of green euglenoid flagellates in light and in darkness has long been of interest. Several species of *Euglena*, including *E. gracilis*, are known to be capable of photoautotrophic nutrition. That is, inorganic compounds suffice for growth in light (Pringsheim, 1912; Mainx, 1928; Dusi, 1933*a*, *b;* Hall, 1938, 1939; Hall and Schoenborn, 1939*a*). Previous reports indicate, however, that such inorganic materials will not support growth in darkness. Of the species studied, only two—*E. mesnili* and *E. gracilis*—have been cultured in the absence of light for any appreciable period, and these in media containing organic nitrogen sources (Lwoff, 1932; Dusi, 1933*a*, *b;* Jahn, 1935; Lwoff and Dusi, 1935). No reports are available on *E. mesnili;* but the studies of Lwoff (1932) and Lwoff and Dusi (1937, 1938) on *E. gracilis* indicate that inorganic nitrogen sources, even in the presence of an organic carbon source, are not sufficient for growth in darkness.

On the other hand, various colorless flagellates have been reported as capable of utilizing inorganic nitrogen when an organic carbon source is supplied (heteroautotrophic nutrition). While several such reports are available for cryptomonad and phytomonad flagellates, it is only recently that the heteroautotrophic nature of a colorless euglenoid, *Astasia* sp., has been demonstrated (Schoenborn, 1940). This colorless organism thus appears to have simpler nutritive requirements than any of the green euglenoids living in the absence of light. However, several factors, such as adaptation and the necessity for favorable oxygen tension, were found to complicate the establishment of *Astasia* in simple media, and it was thought that similar complications may have been responsible for the failure of previous investigators to demonstrate heteroautotrophic nutrition in *E. gracilis*. Preliminary observations (Schoenborn, 1939), tending to confirm this possibility, led to a further study of the problem.

MATERIAL AND METHODS

The bacteria-free strain of *E. gracilis* used in this investigation is the one previously studied by Mainx (1928), Lwoff and Dusi (1937, 1938), Hall and Schoenborn (1939*a*, *b*), and others. This strain was established in pure line by Mainx and forwarded by Professor E. G. Pringsheim to Professor R. P. Hall in 1931. Stock cultures have since been maintained bacteria-free (Hall, 1937).

The experimental media used are shown on the following page.

Most investigators have used test tubes for bacteria-free cultures of Protozoa, but in the present investigation both tubes and flasks were employed. This procedure was adopted after it was found that *Astasia*, in certain media, gave better growth in flask than in tube cultures (Schoenborn, 1940). The method of serial transfers was followed. For any given transfer a certain medium was prepared and the pH adjusted by NaOH

or HCl so that, after sterilization, it would fall within the optimal range for growth of *E. gracilis* (Jahn, 1931; Alexander, 1931; Dusi, 1933*a*). This medium was measured into 16×150-mm. tubes (9.0 cc.) and 125-cc. Erlenmeyer flasks (36 cc.) and sterilized for 20 minutes at 115° C. The tubes then received a 1.0-cc. inoculum from a culture of *E. gracilis*, and the flasks 4.0 cc. Approximately ten tubes and two flasks were inoculated in each transfer. Three of these tubes were fixed for determinations of the initial density of population or initial count (number of flagellates per cubic centimeter), one was used to determine the initial pH, and the remaining tubes and flasks were incubated in the dark for varying periods of time. After the incubation period, three tubes were fixed for the final count, one was used for determination of final pH, and one of the flasks was used to inoculate the tubes and flasks of the next transfer. One of these newly inoculated flasks, after incubation, was then used to inoculate the succeeding transfer, etc.

MEDIUM DJ

NH_4NO_3	0.5 gm.
$NaC_2H_3O_2 \cdot 3H_2O$	1.0 gm.
KH_2PO_4	0.5 gm.
$MgSO_4 \cdot 7H_2O$	0.1 gm.
NaCl	0.1 gm.
$MnCl_2 \cdot 4H_2O$	0.0001 gm.
$FeCl_3 \cdot 6H_2O$	0.0025 gm.
Dist. water	1.0 liter

MEDIUM DI

$(NH_4)_2HPO_4$	1.0 gm.
$NaC_2H_3O_2 \cdot 3H_2O$	1.0 gm.
KH_2PO_4	0.2 gm.
$MgSO_4 \cdot 7H_2O$	0.2 gm.
KCl	0.2 gm.
$FeCl_3 \cdot 6H_2O$	0.0025 gm.
Dist. water	1.0 liter

MEDIUM D

NH_4Cl	0.497 gm.
$NaC_2H_3O_2 \cdot 3H_2O$	1.148 gm.
$MgSO_4$	0.048 gm.
$K_2HPO_4 \cdot 3H_2O$	0.209 gm.
Dist. water	1.0 liter

OTHER MEDIA

Medium EAB: same as medium DJ, but without the sodium acetate

Medium EDA: same as medium DI, but without the sodium acetate

All cultures were incubated in darkness. Both tubes and flasks were first placed in a light-proof copper box, which was in turn placed in a light-proof cabinet or incubator. However, the cultures were exposed to light during inoculation—a process requiring about 10 minutes.

Samples from the tubes fixed at both the beginning and the end of the incubation period were counted by means of a Whipple ocular micrometer and a Sedgwick-Rafter counting chamber (Hall, Johnson, and Loefer, 1935). Thus the population densities of tube cultures listed below are the averages of at least one count from each of three tubes. In flask cultures the initial count should be the same as that of the tubes of the same transfer, since the ratio of inoculum to medium was identical (1:9 and 4:36). The final population density of a flask culture was calculated from the initial count of the tubes inoculated from this flask. An index of the amount of population growth occurring during each transfer can be obtained from the ratio of final (x) to initial (x_0) population density.

The stock of *E. gracilis* used to inoculate Series I–III had already been grown (in light) for twelve successive transfers, covering a period of about eight months, in a medium containing an inorganic nitrogen source and acetate. In the twelfth transfer the calculated peptone concentration was not more than 1.8×10^{-15} gm. per cubic centi-

meter. Thus, even in the first transfers of the experimental series there was no appreciable organic nitrogen source.

Pyrex tubes and flasks were employed, and all glassware was thoroughly washed and steamed before being used. Bleached nonabsorbent cotton was used to plug the tubes, flasks, and pipettes. Chemicals used in the preparation of media were of analytical grade, while the water was double-distilled, the last distillation being made in Pyrex glass. All pH determinations were made with a LaMotte roulette colorimeter. In the experimental results described below only the initial pH values are given, for only rarely did the final reading differ by more than 0.2 from the initial pH.

RESULTS

Several different media were employed in these experiments. If growth does not occur in a given medium, only a few serial transfers are possible, for in each new transfer the flagellate concentration is reduced to one-tenth that of the preceding transfer.

Series I (*medium D*).—Only three transfers were possible in this medium. In these transfers, initial pH was 6.5–6.7. The cultures were incubated at room temperature for 21 days in the first and 42 days in the last two transfers. Results (Table 1) show little or no growth. The population densities decreased, until no organisms were observed in the third transfer. It appears, therefore, that medium D will not support continuous growth of *E. gracilis* in darkness.

Series II (*medium DJ*).—Although this series was carried through nine successive transfers, organisms were observed in the first six only. Initial pH was 6.6–6.7, and incubation periods for the nine transfers were 21, 21, 42, 42, 28, 35, 49, 59, and 57 days, respectively. The first six transfers were incubated at room temperature, the last three in an incubator at 24°–26° C. From the results (Table 1) it can be seen that little or no growth occurred in some of the transfers, while in others there was a decided increase in the number of organisms. In all transfers growth was better in flask than in tube cultures. Even though this medium supported a population for only six transfers, the possibility remains that it is adequate for growth of *E. gracilis* in darkness. For it should be noted that the seventh transfer was not inoculated from a flask culture, as were the other transfers, but from tube cultures. This change of procedure, necessitated by a contamination in the flasks of the sixth transfer, may account for the absence of flagellates in the seventh and subsequent transfers. That is, the sixth-transfer flask cultures may have had higher final populations than the tube cultures; and, had a flask been used to inoculate the seventh transfer, more organisms would have been present initially. Such a higher initial count might have resulted in the continuation of this series. In view of the results obtained concurrently in Series III, it did not seem necessary to retest the suitability of medium DJ, since the main purpose of these experiments was to determine whether *E. gracilis* was capable of heteroautotrophic nutrition.

Series III (*medium DI*).—Incubation periods in the first nine transfers were the same as in series II, while the tenth transfer was incubated for 60 days. Initial pH was 6.7 in all transfers. The first six transfers were incubated at room temperature, the last four at 24°–26° C. As shown in Table 1, little growth occurred in the majority of transfers, but in a few cases there was a marked increase in population density. The results also show that population growth was again better in flask than in tube cultures. Perhaps because of this better growth in flasks, which were always used to inoculate succeeding

transfers, it was possible to carry this series through ten successive transfers covering a period of over thirteen months, and the series is still being continued as stock cul-

TABLE 1

SERIES I–V: GROWTH OF *E. gracilis* IN DARKNESS

The initial and final population densities in the successive transfers are expressed as the number of organisms per cubic centimeter; the "index of growth" as x/x_0, or the ratio of final to initial density of population.

Series	Transfers	Initial Density of Population	Final Density of Population		Index of Growth	
			Tube Cultures	Flask Cultures	Tube Cultures	Flask Cultures
I.....	1	313	203	200	0.6	0.6
	2	20	40	0	2.0	.....
	3	0	0	0*	.....	.....
II.....	1	542	680	800	1.2	1.4
	2	80	156	200	1.9	2.5
	3	20	113	1,130	5.6	56.5
	4	113	1,246	1,500	11.0	13.2
	5	150	236	520	1.5	3.4
	6	52	43	†	0.8	†
	7	0	0	0	.....	.....
	8	0	0	0	.....	.....
	9	0	0	0*	.....	.....
III.....	1	542	680	960	1.2	1.7
	2	96	126	160	1.3	1.6
	3	16	1,350	2,700	84.3	168.7
	4	270	1,173	1,800	4.3	6.6
	5	180	233	300	1.2	1.6
	6	30	23	100	0.7	3.3
	7	10	10	220	1.0	22.0
	8	22	16	50	0.7	2.2
	9	5	40	630	8.0	126.0
	10	63	143	453*	2.2	7.1
IV.....	1	52	36	0	0.6	.....
	2	0	0	0	.....	.....
	3	0	0	0	.....	.....
	4	0	0	0*	.....	.....
V.....	1	30	36	0	1.2	.....
	2	0	7	0	.....	.....
	3	0	0	0	.....	.....
	4	0	0	0*	.....	.....

* Values given are not calculated values but are the results of making direct final counts of flask cultures.

† Calculations not possible, since flasks were contaminated.

tures.[1] It is thus concluded that medium DI is capable of supporting slow growth of *E. gracilis* in darkness.

It will be noted that good growth occurred in the third transfer of Series III, resulting

[1] Thanks are due to Dr. K. L. Osterud for maintenance of the stock after the tenth transfer.

in an increased final density of population; this is true in the third transfer of Series II also, but only in regard to flask cultures. Similar phenomena, previously observed by Hall and Schoenborn (1939*b*) for *E. gracilis*, *E. anabaena*, and *E. viridis* grown autotrophically in daylight, have been interpreted as indicating a selective action of the medium on our strains of *Euglena*.

In addition to the flagellates observed in making initial and final counts, microscopic examinations were made of living organisms taken from various transfers of Series III. These observations revealed no sign of chlorophyll. To check further whether the chlorophyll had been entirely lost during the period of growth in darkness, a group of tubes containing peptone medium was inoculated from ninth-transfer tube and flask cultures and incubated in daylight for several days. At the end of that time they showed a heavy growth of *E. gracilis*, and the organisms were bright green. Evidently, therefore, the chlorophyll is not lost permanently after approximately one year of growth in darkness.

MEDIA WITHOUT ACETATE

After *E. gracilis* had been cultured for several successive transfers in media containing inorganic nitrogen and organic carbon sources, an attempt was made to determine whether the organic carbon source in these solutions was necessary. Series IV and V describe this part of the investigation.

Series IV and V.—Medium EAB (same as DJ but without sodium acetate) served for Series IV, and EDA (DI without acetate) for Series V. Series IV and V were inoculated from the fifth transfers of Series II and III, respectively; hence, the first transfers of Series IV and V are comparable to the sixth of Series II and III, the second to the seventh, etc. All factors (as pH, incubation periods, etc.) in the four transfers of Series IV and V were identical with those listed above for transfers 6–9 of Series II and III. The results (Table 1) show that growth did not occur in either of these purely inorganic solutions, and no organisms were observed in the later transfers. Viability tests were carried out. Tubes containing peptone medium, inoculated from fourth-transfer tube and flask cultures of Series IV and V and incubated for several weeks in daylight, failed to reveal any organisms. It thus appears that under the conditions of the experiment neither medium EAB or EDA is capable of supporting growth of *E. gracilis* in the absence of light. In medium EDA this is evidently due to the lack of an organic carbon source, for, when sodium acetate is added to this medium, growth results (Series III).

DISCUSSION

It is known that purely inorganic media suffice for growth of *E. gracilis* in light (Pringsheim, 1912; Mainx, 1928; Dusi, 1933*a;* Hall and Schoenborn, 1939*a*). As demonstrated above, such media also permit continuous growth in the absence of photosynthesis, provided an organic carbon source is added. Thus, *E. gracilis*, known to be a photoautotroph, may also be regarded as a facultative heteroautotroph.

On the basis of available information it appears that *E. gracilis* has simpler nutritional requirements, even when deprived of photosynthesis, than certain other species of *Euglena* when cultured under conditions which permit photosynthesis. Both *E. deses* and *E. pisciformis* have been reported as incapable of utilizing inorganic nitrogen sources when grown in light (Dusi, 1933*b*; Hall and Schoenborn, 1938). However, *E. gracilis*, as a heteroautotroph, is not unique among the flagellates. One other euglenoid, the colorless *Astasia*, has also been found to be capable of utilizing an inorganic nitrogen source in

the presence of an organic carbon source (Schoenborn, 1940). And several investigators, following Pringsheim's (1921) early report on *Polytoma uvella*, have described heteroautotrophic nutrition for cryptomonad and phytomonad flagellates. However, the failure of other workers to confirm such findings makes it difficult to ascertain the extent of heteroautotrophic nutrition in these groups.

Under the conditions of the present experiments, *E. gracilis* grew only slowly. This may be due, at least partially, to both chemical and physical factors. Medium DI contains appreciable quantities of C, H, O, N, P, K, Mg, S, Na, Cl, as well as traces of As, Cu, Fe, Mn, Ca, Pb, Zn, and Ba. Since *E. gracilis* grew continuously in this medium, the above list must include all elements essential for growth of this flagellate in darkness, unless some necessary element is present as an undetected trace. However, all these elements may not be essential, a few may even be harmful, and it is unlikely that any of them are present in their optimal concentrations. Thus, investigations designed to delimit the essential elements and to determine optimal concentrations may result in better growth of *E. gracilis* in the absence of light. A faster growth rate might also result from investigations dealing with such physical factors as temperature. Jahn (1935), in studying the effect of temperature on growth of *E. gracilis* in darkness, found an optimum of 10° C. in a peptone medium. But this optimum was shifted to 23° C. by the addition of sodium acetate. Our experiments were carried out near the optimum for the acetate-containing medium; however, it is uncertain that the substitution of an inorganic nitrogen source for the peptone will not cause a further shift in the temperature optimum.

The present findings on *E. gracilis* are contradictory to the earlier results of Lwoff (1932) and Lwoff and Dusi (1937, 1938). These workers concluded that peptones are necessary as nitrogen sources in darkness, unless thiamine or its pyrimidine constituent is furnished as a growth factor. When such a growth factor is present, the peptone can be replaced by asparagine but not by an ammonium salt. The present findings indicate that neither the growth factor nor the organic nitrogen source is necessary. Such differences in experimental results are not unusual in the general field dealing with the trophic nature of Protozoa. Although the author has previously discussed some of the factors which may lead to conflicting results (Schoenborn, 1940), those which are directly concerned with the present investigation may be briefly mentioned.

First, there is some indication that flask cultures permit better growth than tube cultures, and, while the differences observed were often not great, they may still be important, especially in view of the usually slow rate of growth. Perhaps it was this better growth in flasks which permitted sufficient serial transfers to justify positive conclusions. It appears that investigators who previously studied the nutrition of *E. gracilis* in darkness have used tube cultures only, and this may account for their negative results.

The occurrence of a selective process may be another factor partially or wholly responsible for previous failures to grow *E. gracilis* on inorganic nitrogen sources in darkness. That selection occurred during the establishment of strains of *E. gracilis*, *E. anabaena*, and *E. viridis* in purely inorganic media was suggested by Hall and Schoenborn (1939*b*). These species, upon being transferred from peptone stock cultures to inorganic media and permitted to grow in light, showed negligible or slow population growth during the first two or three transfers, and then a greatly increased growth rate in the third or fourth transfer. This fact, correlated with certain morphological observations, led to the proposal that some of the organisms in the original peptone strain were in-

capable of photoautotrophic nutrition. These organisms died during the early transfers, those capable of photoautotrophic nutrition survived, and a selected strain resulted. The experimental series in the present investigation were inoculated from such a photoautotrophic strain of *E. gracilis*, and this factor may have been important. Had a peptone strain been used, as was evidently done by earlier workers, a large percentage of the organisms would presumably have failed to survive the early transfers, and this *plus* the low growth rate and the dilution of flagellates inherent in the serial transfer technique may have made the establishment of a strain in darkness more difficult. In addition, a selective or adaptive process may have occurred during the course of the experiments in darkness. There was a greatly increased growth rate in the third transfer of Series II and III; and, although no apparent morphological differences were observed during the early transfers, it is still possible that selection occurred or that the strain became adapted to the new environment. Further studies comparing growth characteristics of the strain in darkness, the photoautotrophic strain, and the peptone strain might provide additional information on this point.

Even the method, that of serial transfers, may be a limiting factor in attempts to demonstrate simple nutritional methods in Protozoa. Unless the growth rate of an organism in a given medium equals or exceeds the rate of dilution occurring at the inoculation of new transfers, the strain will be lost. In this connection the incubation time and the ratio of inoculum to experimental medium are important considerations.

All the above-mentioned factors emphasize the difficulty of drawing conclusions concerning the minimal growth requirements of plantlike flagellates. Until the various possibilities have been checked, negative results may only indicate that certain conditions are unsatisfactory and not that the protozoan in question requires complex organic substances for growth. Perhaps some plantlike flagellates do have such requirements, but conclusions of this nature can be made only after thorough investigations.

LITERATURE CITED

ALEXANDER, G. 1931. The significance of hydrogen ion concentration in the biology of *Euglena gracilis* Klebs. Biol. Bull., **61**:165–84.

DUSI, H. 1933*a*. Recherches sur la nutrition de quelques Euglènes. I. *Euglena gracilis*. Ann. Inst. Pasteur, **50**:550–97.

———. 1933*b*. Recherches sur la nutrition de quelques Euglènes. II. *Euglena stellata, klebsii, anabaena, deses*, et *pisciformis*. *Ibid.*, pp. 840–90.

HALL, R. P. 1937. Growth of free-living Protozoa in pure cultures, in Culture methods for invertebrate animals, pp. 51–59. Ithaca: Comstock.

———. 1938. Nitrogen requirements of *Euglena anabaena* var. *minor*. Arch. f. Protistenk., **91**:465–73.

———. 1939. The trophic nature of *Euglena viridis*. Arch. Zool. expér. gén., **80**:61–67.

HALL, R. P.; JOHNSON, D. F.; and LOEFER, J. B. 1935. A method for counting Protozoa in the measurement of growth under experimental conditions. Trans. Amer. Micr. Soc., **54**:298–300.

HALL, R. P., and SCHOENBORN, H. W. 1938. Studies on the question of autotrophic nutrition in *Chlorogonium euchlorum*, *Euglena anabaena* and *Euglena deses*. Arch. f. Protistenk., **90**:259–71.

———. 1939*a*. The question of autotrophic nutrition in *Euglena gracilis*. Physiol. Zoöl., **12**:76–84.

———. 1939*b*. Selective effects of culture media in bacteria-free strains of *Euglena*. Arch. f. Protistenk., **93**:72–80.

JAHN, T. L. 1931. Studies on the physiology of the euglenoid flagellates. III. The effect of hydrogen-ion concentration on the growth of *Euglena gracilis* Klebs. Biol. Bull., **61**:387–99.

———. 1935. Studies on the physiology of the euglenoid flagellates. VI. The effects of temperature and of acetate on *Euglena gracilis* cultures in the dark. Arch. f. Protistenk., **86**:251–57.

LWOFF, A. 1932. Recherches biochimiques sur la nutrition des Protozoaires. Mono. Inst. Pasteur. Paris: Masson. Pp. 158.

LWOFF, A., and DUSI, H. 1935. La Suppression expérimentale des chloroplastes chez *Euglena mesnili*. Compt. rend. Soc. biol., **119**:1092–95.

———. 1937. Le Thiazol, facteur de croissance pour *Polytoma ocellatum* (Chlamydomonadiné): importance des constituents de l'aneurine pour les flagellés leucophytes. Compt. rend. Acad. sci., **205**: 882–84.

———. 1938. Culture de divers flagellés leucophytes en milieu synthétique. Compt. rend. Soc. biol., **127**:53–56.

MAINX, F. 1928. Beiträge zur Morphologie und Physiologie der Eugleninen. II. Untersuchungen über die Ernährungs- und Reizphysiologie. Arch. f. Protistenk., **60**:355–414.

PRINGSHEIM, E. G. 1912. Kulturversuche mit chlorophyllführenden Mikroorganismen. II. Zur Physiologie der *Euglena gracilis*. Beitr. z. Biol. d. Pflanz., **12**:1–47.

———. 1921. Zur Physiologie saprophytischer Fagellaten (*Polytoma*, *Astasia*, und *Chilomonas*). Beitr. allg. Bot., **2**:88–138.

SCHOENBORN, H. W. 1939. Growth of *Euglena gracilis* on inorganic nitrogen sources in the absence of light. Anat. Rec., Suppl., **75**:151.

———. 1940. Studies on the nutrition of colorless euglenoid flagellates. I. Utilization of inorganic nitrogen by *Astasia* in pure cultures. Ann. New York Acad. Sci., **40**:1–36.

GROWTH OF CHLOROGONIUM TETRAGAMUM AS AFFECTED BY HYDROGEN-ION AND SODIUM ACETATE CONCENTRATIONS

(Five figures)

JOHN B. LOEFER
Berea College

THE importance of regulating pH for successful bacteria-free culture of different species of Protozoa is indicated by a long list of papers pertaining to this subject. Those up to 1935 are listed (Loefer, 1935) in connection with a study of *Chlorogonium euchlorum* Ehrenb. and *C. elongatum* Dangeard. Since then growth of *C. tetragamum* Bohlin in relation to pH has been investigated, and a report of that study follows.

It is well known that sodium acetate is a nutrient for certain species (cf. Lwoff, 1932; Pringsheim, 1921; Loefer, 1935). These investigators used it in concentrations of 0.2 per cent or higher for their experiments. That this amount may be well above the optimum for growth of, and actually harmful to, certain species was shown by experiments on *Paramecium bursaria* (Loefer, 1936), for which species a concentration of 0.1 per cent was toxic and 0.2 per cent lethal. Hall (1937) observed similar effects on the growth of *Euglena stellata*. He noted that 0.05 per cent sodium acetate supported most vigorous growth. Since so little information is available concerning the optimum concentration of sodium acetate which is most favorable for Protozoa, it seemed desirable to investigate this problem as far as *C. tetragamum* was concerned.

MATERIAL AND METHODS

The strain of *C. tetragamum* used was one cultured bacteria-free by Professor E. G. Pringsheim, while in Prague. It was obtained through the courtesy of Professor R. P. Hall from New York University.

The methods used were outlined in a previous paper (Loefer, 1935). Other procedures are given in connection with each experiment. The symbol X is used to designate the final concentration of organisms per cubic centimeter; X_0 represents the concentration per cubic centimeter at the beginning of the experiment. The ratio X/X_0 is used to indicate amount of growth in the tables and figures. The customary tests for purity of the cultures were employed, and all contaminated cultures were discarded.

EXPERIMENTAL RESULTS

Series I: Effect of pH.—The medium used had the following composition: NaCl, 0.003 gm.; $CaSO_4$, 0.015; $MgSO_4$, 0.0045; KNO_3, 0.0013; KH_2PO_4, 0.1; $FeCl_3$, 0.0025; Bacto-tryptone, 10 gm.; and distilled water, 1 liter. Separate lots of this medium were adjusted to different pH values with normal KOH or HCl. Hydrogen-ion concentrations, after inoculation from the stock culture and as indicated in Table 1 and Figure 1, ranged from pH 4.4 to pH 8.5. At least 4–6 tubes of medium of each lot were inoculated and incubated at 28° C. in a constant-temperature water bath under constant light.

The initial concentration of organisms (X_o) was 1,513. Incubation time was 42 hours. The results (Table 1 and Fig. 1) indicate maximum growth in the range of pH 6.8–pH 8.5. Growth at pH 4.4 was very slight compared to that at pH 6.8, 7.1, 7.3, and 7.7, where the organisms numbered around 30,000 and had increased more than nineteen times.

TABLE 1

SERIES I: EFFECT OF pH

(X_o = 1,513; Incubation Time = 42 Hours)

pH	Final Count	X/X_o	pH	Final Count	X/X_o
4.4	1,834	1.21	6.8	29,250	19.46
4.6	3,420	2.26	7.1	29,098	19.21
5.0	8,623	5.78	7.3	30,043	19.82
5.4	11,305	7.47	7.7	30,700	19.86
5.8	20,041	13.50	8.0	27,640	18.50
6.1	21,733	14.31	8.5	25,600	16.90
6.5	23,800	15.71			

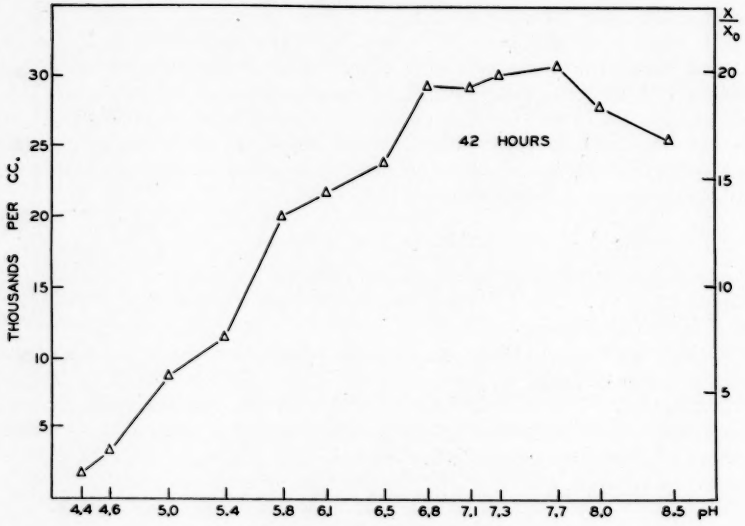


FIG. 1.—Series I: effect of pH

Series II: Effect of pH.—The same medium was used as in Series I. Cultures were established at each of the following hydrogen-ion concentrations: pH 4.4, 5.1, 5.7, 6.2, 6.7, 7.2, 7.8, 8.3, and 8.6. After inoculation from a 48-hour stock, the initial count was 4,620. Some of the cultures of the series were incubated 70 hours; others 95 hours. The results (Table 2 and Fig. 2) indicate best growth in the range pH 6.7–pH 8.6. After 70 hours no increase was apparent at pH 4.4, although survival of some of the organisms

TABLE 2

SERIES II: EFFECT OF pH

(X_0=4,620)

Initial pH	pH—70 Hours	X—70 Hours	X/X_0	pH—95 Hours	X—95 Hours	X/X_0
4.4	4.4	.....	.....	4.4	7,700	1.66
5.1	5.1	8,203	1.77	5.1	12,100	2.61
5.7	5.7	21,307	4.60	5.7	32,666	7.50
6.2	6.2	68,102	14.7	6.2	88,000	19.0
6.7	6.8	143,205	31.0	6.8	235,066	50.8
7.2	7.3	147,233	31.8	7.4	248,400	53.6
7.8	7.9	151,435	32.7	8.0	240,000	52.0
8.3	8.4	149,431	32.3	8.5	225,400	48.6
8.6	8.6	125,326	27.1	8.7	215,500	46.5

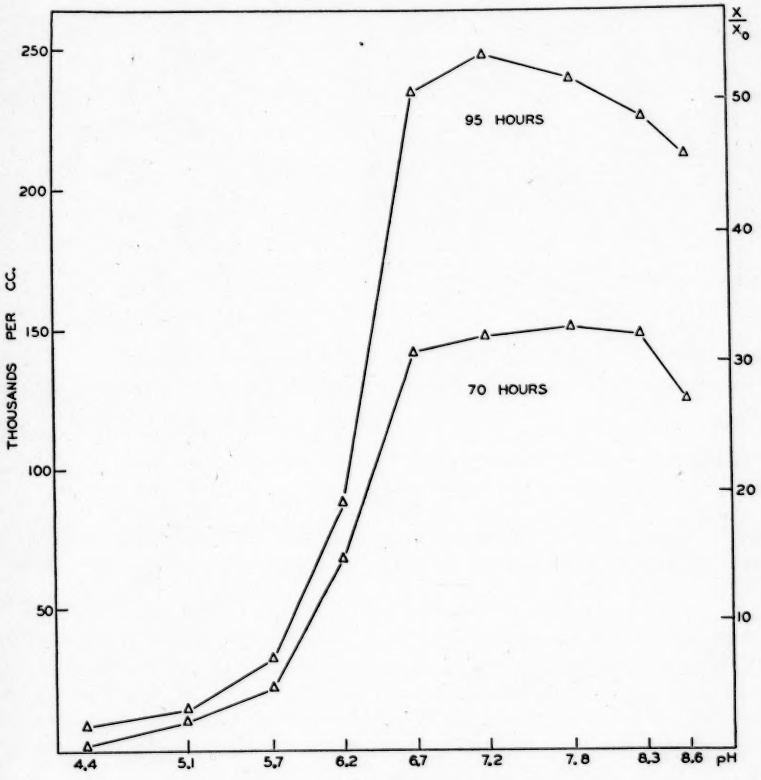


FIG. 2.—Series II: effect of pH

evidently occurred, since after 95 hours they had increased 66 per cent. The highest count observed after 70 hours was at pH 7.8, in which cultures the organisms averaged 151,435 per cubic centimeter. After 95 hours the greatest number of organisms was observed at pH 7.4. Thus at 70 hours the optimum appears to be at a higher pH than at 95 hours. These slight differences, not statistically significant, may be due to a slight shift in pH during the culture period, as one can see by examining the figures in Table 2. At pH 5.1, 5.7, and 6.2, growth was much less than at lower hydrogen-ion concentrations.

Series III: Effect of pH.—The medium for this experiment consisted of: NH_4NO_3, 1.0 gm.; $MgSO_4 \cdot 7H_2O$, 0.2; KH_2PO_4, 0.2; $CaCl_2$, 0.1; $FeCl_3$, 0.0025; Bacto-tryptone, 5.0 gm.; and distilled water, 1 liter. Methods of preparation and inoculation were similar to those used in Series I and II. Cultures were prepared, in which the pH following inoculation was 4.2, 4.8, 5.3, 5.6, 6.1, 6.5, 6.8, 7.1, 7.5, 7.9, 8.1, and 8.5. Slight growth occurred at pH 4.2; and, with lower hydrogen-ion concentrations, growth was progressively better until a maximum count of 20,561 organisms per cubic centimeter was reached at pH

TABLE 3

SERIES III: EFFECT OF pH

(X_o=487)

pH	X—78 Hours	X/X_o	pH	X—78 Hours	X/X_o
4.2	1,261	2.5	6.8	20,561	42.2
4.8	2,803	5.7	7.1	20,349	42.0
5.3	3,951	8.1	7.5	19,567	40.0
5.6	5,030	10.3	7.9	19,000	39.0
6.1	11,407	23.4	8.1	17,648	36.2
6.5	14,031	29.4	8.5	14,204	29.2

6.8. The initial count was 487 and the incubation period 78 hours. Results are shown in Table 3 and Figure 3. An alkalinity of pH 8.5 appears to be somewhat less favorable than in earlier series. Maximum growth occurred in cultures within the range pH 6.8–pH 8.1.

Series IV: Optimum concentration of sodium acetate.—The following medium was used: NaCl, 0.003 gm.; $CaSO_4$, 0.015; $MgSO_4$, 0.0045; KNO_3, 0.0013; KH_2PO_4, 1.0; K_2HPO_4, 1.0; $FeCl_3$, 0.0025; Bacto-tryptone, 10 gm.; distilled water, 1 liter. Hydrogen-ion concentration was adjusted to pH 6.7–pH 6.8. A number of tubes of this medium constituted controls in the experiment. To other portions of this medium, sodium acetate was added to produce concentrations of 0.0125, 0.025, 0.05, 0.1, 0.2, and 0.3 per cent. After tubing and sterilizing, inoculations were made from a 60-hour stock culture growing in medium of the same composition as the control medium. The initial count was 5,910. Incubation was carried out in the same manner as previously, and some tubes were fixed for counting after 35 hours; the others after 45 hours. The results are shown in Table 4 and Figure 4. Examination of these figures indicates that good growth occurred in all the cultures. It was better in those with sodium acetate and best in the medium containing 0.3 per cent sodium acetate. In the heaviest cultures hydrogen-ion changes during the growth period greater than 0.2 pH were not observed.

Series V: Optimum concentration of sodium acetate.—Since maximum growth occurred

with the highest concentration of sodium acetate used (0.3 per cent) in Series IV, it seemed desirable to extend the range of concentrations to determine whether an excess

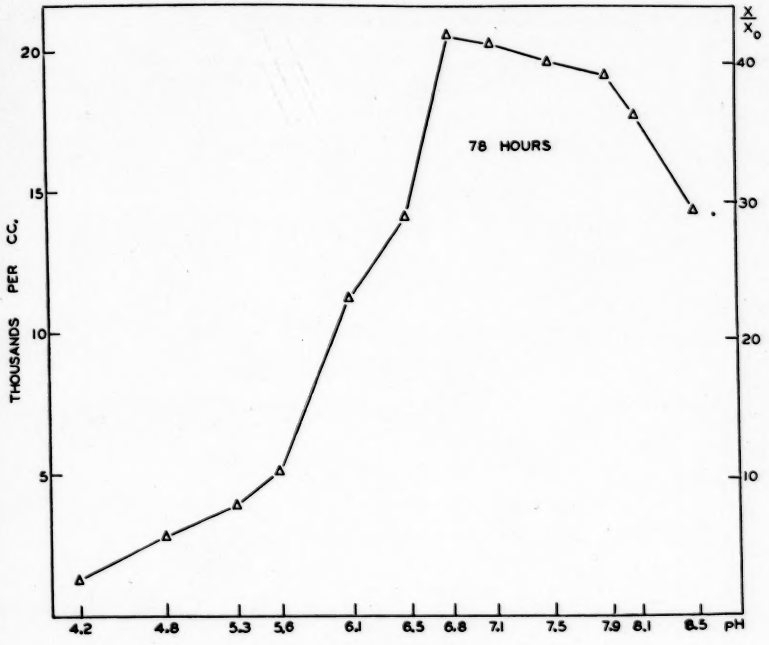


FIG. 3.—Series III: effect of pH

TABLE 4

SERIES IV: EFFECT OF SODIUM ACETATE

(X_0=5,910)

Sodium Acetate Concentration (In Percentage)	X—35 Hours	X/X_0	X—45 Hours	X/X_0	Sodium Acetate Concentration (In Percentage)	X—35 Hours	X/X_0	X—45 Hours	X/X_0
Controls (0)	77,466	13.1	105,500	17.8	0.10	404,623	68.4	416,133	70.4
0.0125	113,866	19.2	149,254	25.2	0.20	501,070	84.7	543,206	91.9
0.025	162,200	27.4	186,400	31.6	0.30	523,666	88.6	572,333	96.8
0.05	229,600	38.8	261,900	44.3					

of sodium acetate is detrimental to growth. Accordingly, media with the following sodium acetate concentrations in the same basic medium as previously used were prepared: 0.125, 0.25, 0.5, 0.75, and 1.0 per cent. The controls contained no sodium acetate. All tubes of the series were inoculated from a 35-hour stock (X_0 = 3,700), and

growth determinations were made after 34 and 70 hours of incubation. The results are shown in Table 5 and Figure 5. It is apparent that all concentrations of sodium acetate tested were beneficial, although the most favorable concentration is around 0.25 per cent. As little as 0.125 per cent is better than 1.0 per cent.

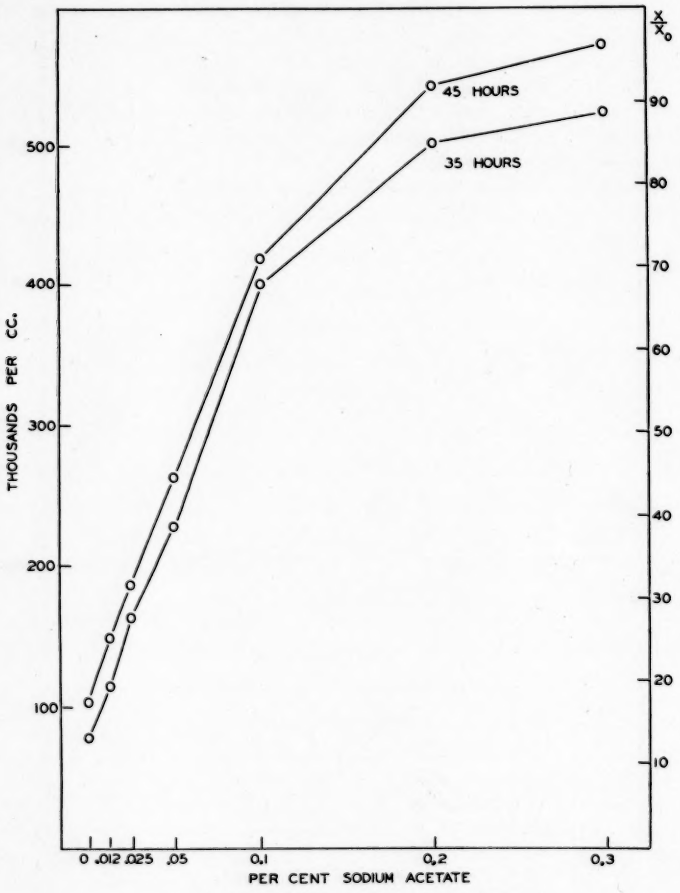


FIG. 4.—Series IV: effect of sodium acetate

DISCUSSION

The relation of pH to growth of Protozoa under bacteria-free conditions was summarized in an earlier paper (Loefer, 1935). Included in this list were the species *C. euchlorum* and *C. elongatum*. Their growth ranges in 0.25 per cent Bacto-tryptone were from pH 4.9 to pH 8.7, with an optimum around 7.4–7.6. Generally speaking, these two phytomonads exhibit maximum growth at a lower hydrogen-ion concentration than

TABLE 5

SERIES V: EFFECT OF SODIUM ACETATE

($X_0 = 3{,}700$)

Sodium Acetate Concentration (In Percentage)	X—34 Hours	X/X_0	X—70 Hours	X/X_0
Controls (0)	102,504	27.7	116,105	31.3
0.125	205,325	55.5	506,250	136.8
0.25	261,224	70.6	813,750	219.9
0.50	233,631	63.1	772,306	208.7
0.75	214,865	58.0	648,050	175.1
1.0	152,636	41.2	446,225	120.6

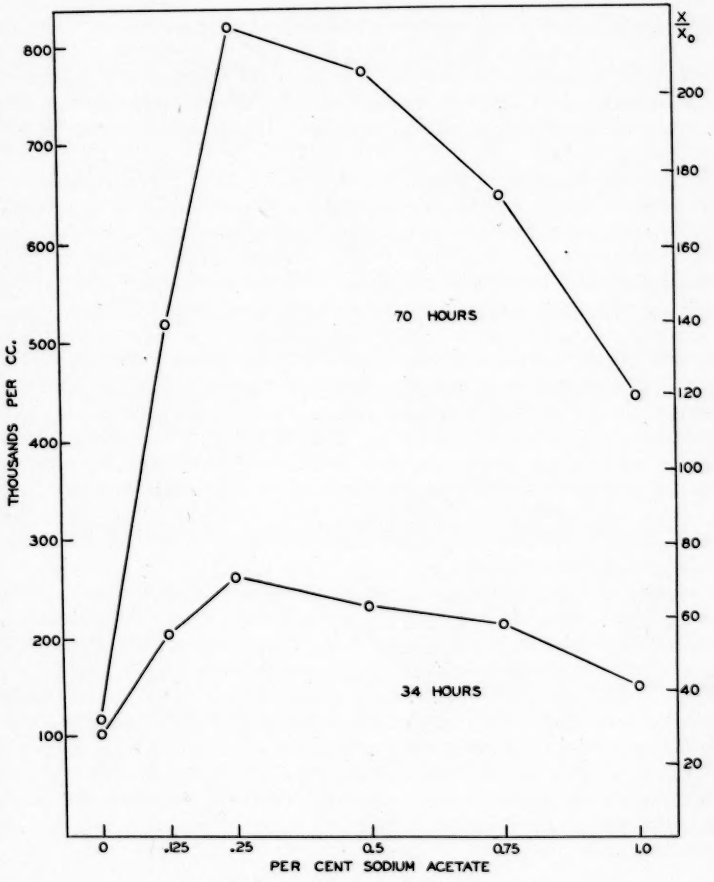


FIG. 5.—Series V: effect of sodium acetate

most species. A preliminary series in a similar medium with *C. tetragamum* (Loefer, 1938*a*) suggested that the maximum pH for this species was even higher. But extended and repeated investigations did not bear this out. *Chlorogonium tetragamum*, however, multiplied at pH 4.2, whereas *C. euchlorum* and *C. elongatum* did not multiply at this pH. This difference may perhaps be due to the fact that the salt content of the culture medium was considerably lower (and perhaps more favorable) than was that in the medium used for the experiments with *C. euchlorum* and *C. elongatum*. The optimum pH range for *C. tetragamum* extends from 6.7 to 8.6, although 8.6 appears to be less favorable than 8.3 in each of the three series. It appears, on the whole, that pH exhibits a similar relationship to growth in the three species of *Chlorogonium*.

Astasia klebsii, according to von Dach (1939, 1940), has an optimum pH range of 4.2–6.0 in a 0.5 per cent Bacto-tryptone medium. The Euglenida, in general, exhibit optimum growth in media more acid than is favorable to *Chlorogonium* (cf. table by Loefer, 1935).

It is interesting to note that *Chilomonas paramecium*, a cryptomonad flagellate, under similar cultural conditions, exhibited two pH-growth maxima. But Mast and Pace (1938) reported otherwise. Their results, quoting them (pp. 431–32), "differ radically. Loefer obtained two optimum hydrogen-ion concentrations, one at pH 4.9 and one at pH 7, whereas we obtained only one and that one (pH 6.8) does not agree with either of those obtained by Loefer."

Closer examination of the results with which they differ reveals that the comparison they made involved growth in noncomparable media. They compared growth in a sodium acetate-containing medium with the growth results obtained by Loefer (1935) in a Bacto-tryptone medium without sodium acetate. Had they compared their results with those of mine in a medium with sodium acetate, as reported in the same paper, their results might not have seemed so radically different. In fact, the findings are quite in agreement, if one merely allows for the limit of error in making colorimetric readings. (Mast and Pace [1937] also compared some results I obtained on growth of *Chilomonas* in an acetate-glucose-peptone salt medium with their findings in an acetate medium containing neither peptone nor glucose.) Thus, while Mast and Pace maximize supposed differences by comparing results in noncomparable media, their findings actually are in agreement with those reported (Loefer, 1935) for comparable media and have been so quoted (Loefer, 1938*b*, p. 181). Similarity of growth maxima in relation to pH values is evident, in spite of the fact that the medium used contained an organic nutrient and a somewhat different proportion of salts than that of the medium used by Mast and Pace.

While sodium acetate has frequently been used in media for Protozoa (Pringsheim, 1921; Lwoff, 1932; Loefer, 1935; Elliott, 1935; von Dach, 1940), its optimum concentration has been determined for a few species only. Pringsheim (1921) observed that concentrations of 0.5 and 0.8 per cent were less favorable than 0.2 per cent for *Polytoma*. Loefer (1936) tested the effect of a series of graded sodium acetate concentrations on *Paramecium bursaria;* but, since this organism apparently did not utilize sodium acetate as a growth substance, only a toxic effect was noted with 0.1 per cent, and as much as 0.2 per cent was actually lethal. Hall (1937) grew *Euglena stellata* with sodium acetate in concentrations ranging from 0.003 to 0.82 per cent and observed that growth was most vigorous in the 0.05 per cent concentration. Toxic effects, especially in light, were evident above 0.2 per cent.

Growth of *Chlorogonium tetragamum* in these experiments was much better in all tested concentrations of sodium acetate than in the controls without any. Even concentrations up to 1.0 per cent (Series V) are beneficial. This species, however, is far more tolerant than *Polytoma* (Pringsheim, 1921), *Paramecium bursaria* (Loefer, 1936), or *Euglena stellata* (Hall, 1937). Although such a high concentration is good, as little as 0.125 per cent is actually better, and concentrations of 0.2–0.5 per cent are best for maximum growth.

SUMMARY

Growth of *Chlorogonium tetragamum* in a 1 per cent Bacto-tryptone salt medium occurred over a range of hydrogen-ion concentrations from pH 4.2 to pH 8.6. Best growth was observed in the media at pH 6.7–pH 8.3. Sodium acetate may be added in concentrations up to 1.0 per cent and result in better growth, but the optimum concentration for the species lies between 0.2 and 0.5 per cent.

LITERATURE CITED

DACH, HERMAN VON. 1939. Population studies on pure cultures of a colorless flagellate, *Astasia klebsii*. Anat. Rec., Suppl., **75**:89.

———. 1940. Factors which affect the growth of a colorless flagellate, *Astasia klebsii*, in pure cultures. Ohio Jour. Sci., **40**:37.

ELLIOTT, A. M. 1935. Effects of certain organic acids and protein derivatives on the growth of *Colpidium*. Arch. f. Protistenk., **84**:472.

HALL, R. P. 1937. Effects of different concentrations of sodium acetate on growth of *Euglena stellata*. Anat. Rec., Suppl., **70**:127.

LOEFER, J. B. 1935. Relation of hydrogen-ion concentration to growth of *Chilomonas* and *Chlorogonium*. Arch. f. Protistenk., **85**:209.

———. 1936. Bacteria-free culture of *Paramecium bursaria* and concentration of the medium as a factor in growth. Jour. Exper. Zoöl., **72**:387.

———. 1938*a*. Growth of *Chlorogonium tetragamum* at different hydrogen-ion concentrations. Anat. Rec., Suppl., **52**:129.

———. 1938*b*. Utilization of dextrose by *Colpidium*, *Glaucoma*, *Chilomonas*, and *Chlorogonium* in bacteria-free cultures. Jour. Exper. Zoöl., **79**:167.

LWOFF, A. 1932. Recherches biochemiques sur la nutrition des protozoaires: le pouvoir de synthèse. Paris: Masson & Cie. Pp. 158.

MAST, S. O., and PACE, D. M. 1937. The relation between hydrogen-ion concentration of the culture medium and rate of growth in *Chilomonas paramecium*. Anat. Rec., Suppl., **70**:40.

———. 1938. The relation between the hydrogen-ion concentration of the culture medium and the rate of reproduction in *Chilomonas paramecium*. Jour. Exper. Zoöl., **79**:429.

PRINGSHEIM. E. G. 1921. Zur Physiologie saprophytischer Flagellaten (*Polytoma*, *Astasia*, und *Chilomonas*). Beitr. z. allg. Bot., **2**:88.

PHYSIOLOGY OF THE CILIATE COLPIDIUM COLPODA. I. THE EFFECT OF VARIOUS BACTERIA AS FOOD ON THE DIVISION RATE OF COLPIDIUM COLPODA[1]

(Four figures)

WILLIAM D. BURBANCK[2]
University of Chicago

ONE trend in contemporary population studies of Protozoa, as recorded in recent extensive reviews (Jahn, 1934; Hammond, 1938; Calkins and Summers, 1941), is toward an analysis of the physiological factors affecting the now familiar Verhulst-Pearl population growth curve, and another is concerned with various growth-promoting and growth-inhibiting substances (Hall, 1941*b;* Johnson, 1941). As would be expected, these studies have tended to become biochemical in nature. In order to reduce technical difficulties in controlling and repeating experiments, Protozoa have been chosen that will grow in either synthetic inorganic or more complex organic nonparticulate media. These studies have made much progress and are important from both a physiological and an ecological viewpoint; however, in the latter field, on account of their relations to interspecific food relations and food chains, studies of interactions between Protozoa and bacteria must not be overlooked.

The standardization of methods for bacterial-protozoan studies involves certain inherent difficulties. Leslie (1940) has described one method of controlling numbers of food bacteria for *Paramecium;* and he, as well as Cleveland (1928) and Hetherington (1934), has shown that the species and strains of food bacteria are very important.

Until recently, holozoic ciliates could be grown only on bacterial or other living particulate suspensions; now, Taylor and van Wagtendonk (1941) have grown *Colpoda* in a medium which consists of an aqueous extract of brewers' yeast and the plasmoptysate of a marine strain of the bacterium *Pseudomonas fluorescens.* Such work may lead away from bacterial-protozoan studies; yet the use of such media will be valuable for maintaining standard controls and stock cultures for further work on bacterial-protozoan relationships.

When bacteria are used as a source of energy for Protozoa over a period of time in mass cultures, certain internal uncontrolled and unanalyzed changes may take place (Butterfield *et al.*, 1931). In growing large protozoan populations on certain bacteria a division rate may be established, but this rate is actually the net gain. Cutler and Crump (1923) found in their work on *Colpidium*(?) that even in the period of greatest reproduction some of their animals were dying. To obtain a close approximation of the reproduction potential of Protozoa the biotic effects of intraspecific competition between Protozoa, and between bacteria and Protozoa should be removed, and, in any event, must be kept at a minimum. It is extremely difficult to eliminate all such competition, since, as

[1] I am greatly indebted to my adviser, Professor W. C. Allee, for generously expending his time and counsel on many aspects of this problem.

[2] Present address: Department of Biology, College of the City of New York.

Kidder and Stuart (1939) have shown, even higher bacteria do multiply in so-called "non-nutrient media."

The isolation small-volume culture technique has been in and out of favor among protozoölogists for many years. This technique lends itself ideally to the study of bacterial-protozoan relations, for, if animals are removed from a stock culture in a phase of most rapid division, they may be so maintained in this phase by daily transfers into freshly prepared isolation cultures (Phelps, 1935; Richards, 1941). In this way the environment is not limited, the food possibilities are not exploited in so short a time, and accumulated waste products do not become a problem. On the other hand, the surface-volume relations differ greatly from those in large-volume cultures.

Hetherington (1934) pointed out that the interesting holozoic ciliate *Colpidium colpoda* has been erroneously described as the experimental animal used by several investigators (Peters, 1921; Cutler and Crump, 1923; Butterfield *et al.*, 1931), while Hetherington (1933, 1934) and Chatton and Chatton (1927*a*, *b*) seem to have actually experimented with this species. As shown by Sandon (1927), this animal is ubiquitous; and it is of further interest because the other good species in the genus, *C. campylum*, can be grown on nonparticulate organic media. Therefore, here is an animal which is at least taxonomically intermediate between the smaller saprozoic forms and the larger holozoic types. Finally, Kidder and Diller (1934) have found no complicated endomixis taking place in the animal; so it, indeed, may be the protozoan par excellence for use in isolation-culture studies.

With this background in mind, *C. colpoda* was used as the experimental animal that was grown on many different monoflorae of bacteria. It should, however, be stated at the outset that this is a survey of a subject which has many ramifications and possibilities. In order to obtain an approximately complete analysis of the interaction between *C. colpoda* and bacteria *x*, *y*, *z*, for example, several strains of both the particular protozoan and the particular bacterium should be used simultaneously, and independent and numerous readings should be taken over a relatively long period of time. This is not the task for one person; the methods adopted were, at least, adequate for a one-man reconnaissance only.

MATERIAL AND METHODS

The ciliate *C. colpoda*[3] was isolated in the winter of 1939 from a *Parmecium* culture supplied by the General Biological Supply House, Incorporated, of Chicago, Illinois. The culture containing these animals had been originally collected from an unrecorded pond in the Chicago region.

Colpidium colpoda was first placed unwashed into a suspension of the bacterium *Aerobacter aerogenes*. Later a permanent stock was established by washing the animals in sterile 0.3 per cent Difco yeast, according to Parpart's method (Parpart, 1928), and the sterile animals were reinoculated into an *A. aerogenes* suspension made up in sterile artificial pond water and kept in a 125-ml. Erlenmeyer flask. This stock and all experimental and control animals were maintained at $25°5 \pm 0°5$ C. Unwashed stock animals were transferred to a fresh bacterial suspension once a month throughout the course of the series of experiments, which lasted for over a year.

The experimental work was divided into two parts. First, *C. colpoda* grown on

[3] I wish to thank Professor Harold Kirby, Jr., of the University of California, for confirming the identification of this protozoan.

A. aerogenes was used as a control, while thirty-one species of the Eubacteriales, belonging to fifteen genera and six families, were tested as food organisms. Second, the same procedure was repeated with the control animal grown on *P. fluorescens*.

As this work is a preliminary phase of an ecological laboratory study of the population of *C. colpoda*, only aquatic or ubiquitous bacteria were used. Of the fifteen genera studied, eight were found to be present in fish-conditioned water used in this laboratory.[4] The only other genus found in this water was *Achromobacterium*, which was not worked with because of the uncertainty of the taxonomy in this group. *Bergey's Manual of Determinative Bacteriology* (1939) was the taxonomic authority in this work; and, when at all possible, an attempt was made to use the type species of any particular genus as one of the organisms tested in that genus. The bacteria used were obtained from the collcction of cultures of the department of bacteriology of the University of Chicago and from the American Type Culture Collection in Washington, D.C.

All bacteria used as food for *C. colpoda* were first transferred to veal-infusion slants and incubated at their optimal temperatures for growth. After 24 hours each slant was washed with 1 ml. of sterile artificial pond water, and growths from three slants were transferred to Kolle flasks containing veal-infusion agar. These, in turn, were incubated for 24 hours at the correct temperatures; then, 5 ml. of sterile artificial pond water were added, and this water was swirled for several minutes with the flask in a horizontal position. No mechanical means were employed to loosen the bacterial growths, with the exception of growths of *Micrococcus conglomeratus* and *Bacillus mycoides*, which adhered tenaciously to the surface of the medium. When a suspension of the bacteria had been obtained, it was added to 25 ml. of sterile artificial pond water. A check of the viable bacteria was made by the dilution method. After this count a stock suspension of the bacteria was prepared such that it approximated the arbitrary number of 150,000,000 viable bacteria per milliliter. These stocks were checked immediately by the dilution method of the beginning of the 10-day period and again at the end of the experiment. The bacterial stock suspensions, which were generally between 90 and 100 ml., were kept for the 10-day period in a cold room at about 6° C.; and daily 0.2 ml. of the suspensions were dispensed to each experimental and control line. The final count showed that the number of viable bacteria remained constant over the 10-day period. The vibrios micrococci, and several spore-formers had to be used at lower dilutions than the one stated above, since with these forms such magnitudes of viable bacteria were so opaque that the Protozoa could not be seen.

At the beginning of a series of experiments single animals were taken from the stock culture and placed in 0.2 ml. of *A. aerogenes* suspension, which had been pipetted into a sterile hard-glass watch glass placed in a sterile Petri dish containing a few milliliters of distilled water. An attempt was made to take animals from the stock culture at the phase of most rapid division or, if this was not possible, to subculture them until they were dividing at a maximum division rate. In the first experimental run of both the *P. fluorescens* and the *A. aerogenes* series, experimental animals were taken from mass cultures; and after that all experimental animals were taken from isolation-culture controls of a previous run. It will thus be seen that the animals used in each series were from cultures grown on the same bacterium that was used in growing the controls for that particular series. Because of unavoidable interruptions, two exceptions existed in the *A. aerogenes* series: animals had to be kept in mass culture during the months of October

[4] By private communication with Mrs. Murvel Garner.

and January, when no experiments were run; but, as mentioned above, these animals were not used for experiments in November and February, respectively, until they were again dividing at a high rate.

When an isolated animal had divided several times, one or several animals were subcultured singly on *A. aerogenes* as unwashed controls, and the rest were washed with five washes of sterile 0.3 per cent yeast extract. After remaining in the fifth wash for 3–5 hours the experimental animals were again washed five times (Parpart's method). Animals were taken from the last wash (No. 10) and placed in the desired bacterial suspensions. The last wash medium was incubated for 48 hours as a test of sterility, and only animals were used that proved to be sterile according to this test. There was found to be no statistical difference between division rates of washed and unwashed animals.

After the experimental and control animals had increased to at least four individuals, these animals were then used to start four lines, which were grown in isolation cultures for a 10-day period with daily subcultures of one animal per line. In subculturing animals a consistent attempt was made to take at random the animals to be transferred. Dividing individuals, or those having just divided, were not used if other individuals not in these stages were available. During the 10-day period, 24-hour counts were made with the aid of a dissecting microscope and a mouth-controlled micropipette. In all individual experiments actual counts, not estimations, of the animals produced in 24 hours were made.

With a few exceptions (see Figs. 1 and 2) the experimental unit used during the 10-day experimental period consisted of two experimental sets of four lines each and one four-line control, all of which were kept in one large crystallizing dish. The cultures were prevented from evaporating by the moisture from several thicknesses of water-saturated filter paper placed in the bottom of the chamber. All the watch glasses containing Protozoa were placed in a regular and consistent order on a circular metal rack and kept from slipping from position by rubber washers attached to the top of the rack. When the Protozoa were to be transferred, the watch glasses were placed in sterile Petri dishes to facilitate handling.

To preclude any contamination while making transfers, a dustproof box[5] was used. This was fitted with a transparent cellulose-acetate spectacle-shaped shield (Plastocele) attached to an upper central opening of the box with oiled silk. The shield fitted over the oculars of a dissecting microscope, making it possible to handle the animals within the box with a minimum danger of contamination. The top and back of the box were glass, and the front and the side inlets were closed with oiled silk "zipper" curtains. During the experimental period the box was washed several times with 70 per cent alcohol, and the dust-free dissecting microscope was used only for transfers. When not in the transfer box, the microscope was kept under a bell jar. Animals were transferred with small sterile mouth-controlled micropipettes. Before the operator's hands were introduced into the box, they were always washed with 70 per cent alcohol.

Hydrogen-ion concentration tests were made on the experimental and control bacterial suspensions with the Hellige method before, during, and after an experimental period. All bacterial suspensions used were made up in unbuffered, sterile artificial pond water, which was developed in this laboratory by Allee *et al.* (1934) and contained per liter:

[5] I wish to thank my father, the Rev. Mr. G. G. Burbanck, for the construction of the dustproof transfer box.

100 mg. $CaCl_2 \cdot 4H_2O$	50 mg. K_2SO_4
50 mg. $MgSO_4 \cdot 7H_2O$	50 mg. $NaNO_3$

All water used for making up the artificial pond water was Pyrex-redistilled water with an arbitrary lower limit of goodness of 2.00×10^{-6} reciprocal ohms.

As a check for bacterial contamination, Gram's stains were run on the bacterial suspensions before they were used and from the last 0.2-ml. cultures in which the experimental and control animals had been living. In only one case out of sixty was any contamination discovered. The results from this experiment were discarded, and the work was repeated. The dilution plates used for checking viable bacteria were also observed for contaminating colonies. For sterilization and handling of cultures and media the usual bacteriological techniques were employed, and methods for wrapping glassware and sterilization followed Buchsbaum and Loosli (1936). All bacterial media used were supplied by the department of bacteriology of the University of Chicago.

TABLE 1

RECORD OF A TYPICAL EXPERIMENTAL UNIT

I. *Salmonella paratyphi* A.

Experimental Subcultures	May 9	10	11	12	13	14	15	16	18	Mean Div.
A	4	4	4	6	4	6	4	3	4	6	2.1340
B	6	4	5	7	4	7	4	5	5	4	2.3166
C	2	8	4	7	4	4	4	5	4	4	2.1129
D	4	2	5	4	7	4	4	4	4	4	2.0129
Mean of means											2.1441

II. *S. schottmulleri*

Experimental Subcultures	May 9	10	11	12	13	14	15	16	17	18	Mean Div.
E	4	8	6	8	8	4	6	3	7	4	2.4562
F	7	8	6	4	4	5	8	7	7	4	2.5329
G	4	8	4	5	8	3	6	5	4	4	2.2814
H	8	6	4	4	4	4	6	4	6	4	2.2755
Mean of means											2.3865

III. *P. fluorescens*

Control Subcultures	May 9	10	11	12	13	14	15	16	17	18	Mean Div.
I	8	8	6	8	7	4	5	3/*k*	5	8	2.5621
J	8	8	8	14	4	8	8	3/*k*	4	8	2.7392
K	7	8	8	11	4	7	4	8	4	8	2.7074
L	8	8	8	8	8	8	7	8	8	8	2.9807
Mean of means											2.7474

PRESENTATION OF DATA

Table 1 shows the results from a typical experimental run. It will be seen that the procedures follow, in a modified form, those Calkins (1933) employed in his studies on protozoan vitality. Fewer lines were used in this work, and no running record of the

number of generations was kept. Parts I and II of the table show the divisions of *C. colpoda* when feeding on *Salmonella paratyphi* A. and *S. schottmulleri*, respectively, while Part III gives the results on the control organism, *P. fluorescens*. The different protozoan subcultures are designated by letters of the alphabet. On May 16 the two subcultures, I and J, contained three animals each which were abnormal for some unknown reason. Since experience had shown that subculturing such abnormal animals would unduly affect the mean of the means for the whole run, as these animals would either die or remain undividing for days, whenever such abnormal individuals appeared, or if there had been a death on the previous day, an individual was taken from another line to keep four lines going for the whole 10 days. It was necessary to follow such a procedure in only 10 per cent of all of the experimental and control runs.

In analyzing the data the actual number of animals produced in 24 hours had to be converted into the equivalent number of divisions. Since the reproduction of *C. colpoda* was exponential, the conversion was made by using the logarithm of the number of individuals per day per line to the base 2. Then the means were obtained which appear at the end of each line in Table 1. The mean of these division rates is given below the four averages mentioned and is the mean division rate for four lines of *C. colpoda* for a 10-day period.

The crux of this problem is whether different species of bacteria, when used as food for *C. colpoda*, significantly change its division rate. Since Protozoa, as with all biological systems, and unlike chemically pure reagents, are highly variable, it is necessary in such problems to accompany experimental tests by a standardized control with which cross-comparisons can be made. In the past such comparisons have usually been made merely by inspection of the mean division rates without subjecting these means to further statistical analyses. With small samples it is generally realized that the statistical significance is limited to the set of data which is being analyzed. In the present instance it is recognized that the experimental tests on any given bacterial species have necessarily been limited in number and that further testing might conceivably change the trends which are indicated in the present work. Even so, the use of statistical analysis of the significance of the differences between mean values, if not overgeneralized, serves as an advance over estimates of significance based upon examination of the means alone.

In the analysis of the present work the method used to test the statistical significance of the observed differences was essentially that developed by "Student" (1925) and by R. A. Fisher (1925).[6] A conservative method was used in the calculation of the *P*-values. As described above, mean division rates were obtained for both experimental and control

[6] Treating the means of four experimental and four control lines provisionally as one homogeneous population, the following formula for t may be used:

$$t = \frac{(\bar{v}_1 - \bar{v}_2)\sqrt{N}}{\sigma_{\text{Total}}},$$

when

$$\sigma_{\text{Total}} = \sqrt{\frac{\Sigma v^2 - \frac{(\Sigma \bar{v})^2}{N}}{N - 1}},$$

$\bar{v}_1$ is the mean of means of the control, and $\bar{v}_2$ is the mean of means of the experiment. $\bar{v}$ is the grand mean, and v is the number of a single control or experimental line. N is the number of control and experimental lines.

TABLE 2

DIVISION RATES OF *C. colpoda* GROWN ON THIRTY-ONE SPECIES OF THE EUBACTERIALES

Bacterial Species Used	Mean 10-Day Division Rate for Four Lines	*P*
I. Rhizobiaceae:		
Chromobacterium violaceum	Toxic	
1. *Ch. viscosum*	2.1080	0.0008
*P. fluorescens**	3.0845	
2. *Alcaligenes fecalis*	1.5439	0.0016
P. fluorescens	2.7424	
3. *Al. viscosus*	2.5467	0.0106
II. Pseudomonadaceae:		
4. *Vibrio comma* (survived 5 days)	0.3000	0.0012
P. fluorescens	3.0161	
5. *V. metchnikovii* (survived 4 days)	0.2500	0.0012
P. fluorescens	3.0315	
6. *P. aeruginosa*	1.0708	0.0012
P. fluorescens	2.5578	
7. *P. fluorescens*	2.7500	0.0182
III. Micrococcaceae:		
8. *M. luteus*	0.3646	0.0012
P. fluorescens	2.9876	
9. *M. conglomeratus*	1.3378	0.0012
P. fluorescens	3.0922	
10. *Staphylococcus aureus*	1.8825	0.0017
11. *St. citreus*	0.4250	0.0012
12. *St. albus*	1.8844	0.0017
P. fluorescens	2.9224	
13. *Sarcina flava*	1.2085	0.0012
P. fluorescens	3.0922	
14. *Sar. lutea*	0.3875	0.0012
IV. Enterobacteriaceae:		
15. *Escherichia coli*	2.1414	0.0016
P. fluorescens	2.8094	
16. *A. aerogenes*	2.9322	0.0622
P. fluorescens	2.8615	
17. *A. cloaceae*	2.3784	0.0016
18. *Serratia marcescens*	2.4405	0.0334
P. fluorescens	2.5941	
19. *Ser. plymouthensis*	2.7848	0.0228

* As in this case, whenever *P. fluorescens* appears unnumbered, it is the basic food organism for control *Colpidium*.

TABLE 2—*Continued*

Bacterial Species Used	Mean 10-Day Division Rate for Four Lines	*P*
20. *Proteus vulgaris*	2.7315	0.0196
P. fluorescens	2.9988	.....
21. *Pr. mirabilis*	2.1077	0.0016
22. *S. schottmulleri*	2.3865	0.0038
P. fluorescens	2.7474	.....
23. *S. paratyphi* A.	2.1441	0.0034
24. *Eberthella typhosa*	1.8321	0.0016
P. fluorescens	2.8094	.....
V. Bacteriaceae:		
25. *Flavobacterium synxanthum*	1.5015	0.0020
P. fluorescens	2.4471	.....
26. *F. acetylicum*	1.7829	0.0030
VI. Bacillaceae:		
27. *B. subtilis*	0.0750	0.0012
P. fluorescens	2.6093	.....
28. *B. mesentericus* (survived only 1 day after several unsuccessful attempts to start it)	.....	.....
29. *B. mycoides*	0.2750	0.0012
P. fluorescens	2.6298	.....
30. *B. megatherium*	1.3646	0.0012

lines; then the four mean division rates of *C. colpoda* grown on a given bacterium and the means from the four accompanying control lines were provisionally treated statistically as one homogeneous population. If, then, the difference between the means of the experimental and the control lines gave, by the *t*-test, a *P*-value greater than the 0.05 level of significance, it was assumed that there was no significant difference between the experimental and the control division rates. However, if the *P*-value was smaller than 0.05, the difference of the division rates between *C. colpoda* grown on the experimental bacterium and the control species (*A. aerogenes* or *P. fluorescens*) was accepted as a real one, i.e., the control and the experimental animals statistically belonged to different populations; and, under the conditions tested, this difference was considered to be due to some quality or qualities of the experimental bacterium. This difference was even apparent, in all but a few cases, when the level of significance was changed from 0.05 to the much lower, but sometimes employed, level of 0.01.[7]

To facilitate the understanding of the experimental results, Table 2 has been arranged with the experimental bacteria grouped according to families. For ready reference the experimental bacteria were numbered in Arabic and the families with Roman numerals. In this series of experiments *Colpidium* grown on *P. fluorescens* was the control. When the experimental animal was grown on a single bacterial species of a genus, that fact was recorded by a single name entered into the table, with its control placed directly below

[7] I am indebted to Dr. Earl L. Green, of Ohio State University, for suggestions on handling the statistics used in this paper.

it; also, the two species in two genera (*Vibrio* and *Micrococcus*) have their controls below each species name. Since three staphylococci were run at one time, their control is directly below their several names. With these exceptions, the name and record of each control was placed between the names and records of each of its experimental bacteria, since they were run at the same time as shown in Table 1. The mean 10-day division rates of *C. colpoda* grown in experimental and control bacterial suspensions are found immediately to the right of each bacterial name, while the *P*-, or probability, value in every case refers to a simple statistical comparison of each experimental performance of *Colpidium* with its own control. It can be ascertained from Table 2 that the division rates of the experimental animal were, in all but a few cases, radically different from their control; i.e., *P* is smaller than the arbitrary value of 0.05.

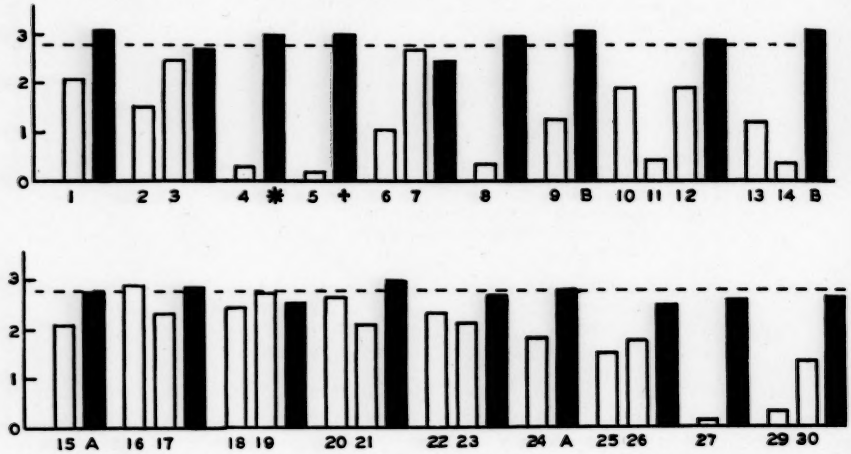


FIG. 1.—The white bars show the average 10-day division rates of *C. colpoda* grown on different species of bacteria; the black ones show the accompanying rates when grown on *P. fluorescens*. Ordinates show division rates; abscissae give the numbers assigned to bacteria in Table 2. The broken line gives the mean for all controls. Controls marked by the same letters are identical. *Five days. +Four days.

The relationships of *C. colpoda* grown on different bacterial species are more strikingly set forth in Figure 1 than in tabular columns of figures. Although all experiments could not, of course, be carried on at one time, a comparison of the black control bars with the broken line indicates that the division rates of the controls were quite constant. In the case of the vibrios, their controls had two of the three highest division rates, so that the poor showings of *Colpidium* on these bacteria were certainly not due to weak experimental animals, since the control and experimentals came from the same stock. A comparison of each pair of white experimental bars indicates that, besides differing from their controls, the different species of bacteria in the same genus supported division rates of *C. colpoda* that were different.

As stated earlier, *C. colpoda* was also grown on a series of thirty-one species of bacteria with *A. aerogenes* as food for the control animals. Because the resulting data were handled in the same manner as represented in Table 2, they were not included in a sepa-

rate table but are given in Figures 2 and 3, which, together, adequately present the experimental results.

Certain significant comparative data can be obtained by careful examination of Figures 1 and 2. On *P. fluorescens* (Fig. 1) the control ciliates had an average division rate that was much higher than when they were grown on *A. aerogenes*. Also, the rate of growth on the controls was more uniform on the former, as indicated by a comparison of the broken lines with the black bars in Figures 1 and 2. The black control bars are more numerous in Figure 2 than in Figure 1, owing to the fact that more bacteria were tested singly against their *A. aerogenes* controls, since all the experimental bacterial cultures were not available at the beginning of the *A. aerogenes*-controlled experiments. The highest rate of division among the controls shown in Figure 2 is that of the control for *Ch. viscosum*. This division rate is higher than that of any for *C. colpoda* grown on

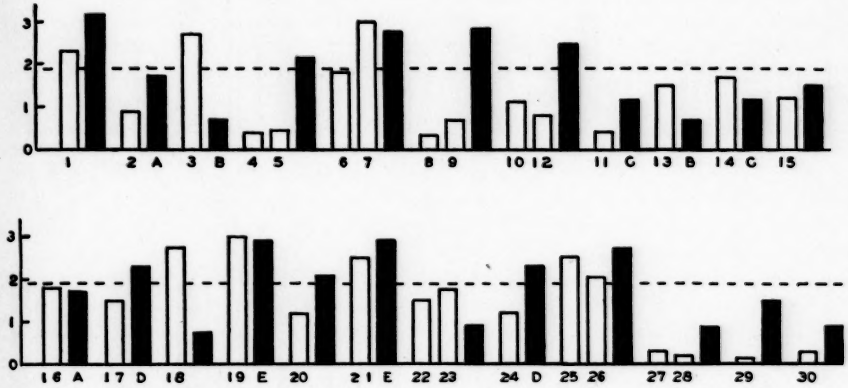


FIG. 2.—The white bars show the average 10-day division rates of *C. colpoda* grown on different species of bacteria; the black ones show the accompanying rates when grown on *A. aerogenes*. Ordinates show division rates; abscissae give the numbers assigned to bacteria in Table 2. The broken line gives the mean of all controls. Controls marked by the same letters are identical.

P. fluorescens. In spite of the unusually high division rates which *A. aerogenes* was capable of supporting, the average division rate of these control animals was lower than that for those grown on *P. fluorescens*. In general, the animals which had been taken from *P. fluorescens* suspension divided at a higher rate but did not necessarily yield experimental animals with higher experimental division rates than those taken from *A. aerogenes* cultures. For example, *Ch. viscosum* (3), *Sar. lutea* (14), and *Ser. marcescens* (18) in Figure 2 all have controls which have lower division rates than those of the corresponding experiments shown in Figure 1; nevertheless, the foregoing three experimental bacteria supported higher division rates of *C. colpoda* than the same bacteria in Figure 1. *Bacillus mesentericus* appears as an experimental bacterium in Figure 2 but not in Figure 1, because it was unable to support *Colpidium* taken from *P. fluorescens* stocks. The two species of vibrios were able to support the animals taken from *P. fluorescens* stocks for only a few days, although animals obtained from *A. aerogenes* suspensions survived on the vibrios for the entire 10-day period. In both Figure 1 and Figure 2 it can

be seen that different species of bacteria belonging to the same genus differ in their ability to support *C. colpoda*, whether obtained from *P. fluorescens* or from *A. aerogenes* stocks. This point and others which concern the comparison of the data in Figures 1 and 2 are discussed in more detail in the following paragraphs.

Although Figure 3 does not help to explain the erratic division rates of *Colpidium* grown on *A. aerogenes*, its chronological arrangement does clearly illustrate a rather extensive period of lowered reproduction. The division rate of *C. colpoda* grown on *A. aerogenes* as the control bacterium started in August, 1940, with a fairly high rate, which continued near or above the average through December. In February, however, division rates became low, increased again in late March, and remained high until the end of

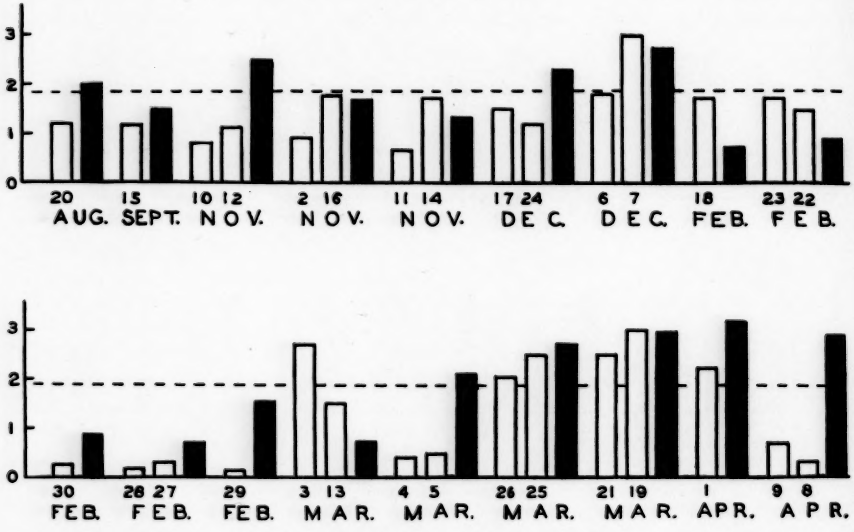


FIG. 3.—A chronological arrangement of Fig. 2

that whole experimental series. That these effects were caused by *A. aerogenes* rather than by some internal change in the animal itself is shown, in Figure 3, by the fairly high division rates of *Colpidium* grown on *Al. viscosus* (3) and *Ser. marcescens* (18), which had division rates more than twice as great as those of their control animals, although both control and experimental animals had recently come from the same culture. There is no explanation for the remarkable March and April recovery of *C. colpoda* controls grown on *A. aerogenes*. However, it was noted that *A. aerogenes* underwent an autoagglutination that regularly appeared on the fifth or sixth day in the 90-ml. stocks kept at 6° C. Hetherington (1934) believed that this in some way caused the lowered division rates. As agglutination consistently occurred in all stock suspensions, this phenomenon probably has no effect on these "epidemics," as the periods of reduced rates of reproduction were called by Hetherington.

During the runs numbered 10, 11, 12, 16, and 17 in Figure 3 *Colpidium* in the control and occasionally in the experiment produced one or two aberrant individuals per day

per line. Only normal animals were subcultured, but still a few abnormal types were produced throughout these runs. It appeared that these bizarre forms were a result of some upset in the time of division, for perfectly formed doubles were found with the posterioanterior ends attached. From the violent pulling that these double monsters exhibited, it was evident that, had they pulled apart, one would have had a mangled posterior and the other a misshapen anterior end. These peculiar malformations were noted in only the particular experiments here cited, and the conditions for these particular tests were the same as for all other experiments, as far as it was possible to determine. No such performances of *Colpidium* were noted at all in the series of experiments based on *P. fluorescens*.

It will be noted in Table 2 that *P. fluorescens* (7) contained washed *C. colpoda* which had an average division rate for a 10-day period of 2.7500, and the control had a *significantly different* division rate of 2.5578. Although this is contrary to an earlier statement (p. 345), this discrepancy can readily be explained. During the last 9 days of the experiment washed versus unwashed animals were compared, and no significant difference was found. In addition, the controls for *E. coli* (15) and *A. cloacae* (17), which were run at the same time, were not significantly different from the washed *C. colpoda* grown in the bacterial suspension common to these three, i.e., *P. fluorescens*. It was found upon reference to laboratory notes that the lower division rate of 2.5578 was due to the few divisions of the control animals on the first day; and this, in turn, was found to have been caused by the slightly different times of divisions of the control and experimental animals before the start of the experimental run. The four animals for the control were taken from a culture which contained seven individuals, while those used for the experiment were taken from one which contained eight animals. This, when interpreted, means that the control animals had not yet finished their third division, while the experimental animals, which had come from one washed animal on the previous day, were beginning on their fourth. Therefore, it may be concluded that the earlier statement is justified in supposing that washing animals causes no significant difference in the subsequent division rate. Fortunately, this case is unique, but it does bear witness to the sensitivity of the system. Otherwise, in experiments in which *P. fluorescens* was used as the control and basic food organism (Table 2), only *C. colpoda* grown on *A. aerogenes* and those grown on *Ser. plymouthensis* had division rates which were higher than those of the accompanying control series. The difference in the case of the former falls above ($P = 0.0622$), and in the case of the latter below ($P = 0.0228$), the stated upper limit of statistical significance. *Serratia marcescens* and *A. cloaceae* supported division rates of *C. colpoda* which were somewhat less than that of their accompanying control with $P = 0.0334$ and $P = 0.0016$, respectively.

When the mean 10-day division rates of *C. colpoda* given in Table 2 were tested statistically (Table 3), it was found that, with the exception of *St. aureus* and *St. albus* (Table 3), different bacterial species of the same genus had significantly different growth-promoting powers. If these data are verified by a more extensive series of tests, this would open an interesting line of study for students of speciation.

In the series in which *A. aerogenes* was the basic food and the food organism for the control series (see Fig. 2), several species supported significantly faster division rates than those shown in the accompanying controls; they were as shown on page 355. All the other experimental bacteria supported fewer divisions of *C. colpoda* than those shown in the accompanying controls. *Serratia plymouthensis* was the only experimental

TABLE 3

DIVISION RATES OF *C. colpoda* GROWN ON DIFFERENT BACTERIAL SPECIES OF THE SAME GENUS

Bacterial Species Used	Mean 10-Day Division Rate for Four Lines	*P*
I. Rhizobiaceae:		
1. *Ch. violaceum*	Toxic	.....
2. *Ch. viscosum*	2.1080	
3. *Al. fecalis*	1.5439	0.0020
4. *Al. viscosus*	2.5467	
II. Pseudomonadaceae:		
5. *P. aeruginosa*	1.0708	0.0012
6. *P. fluorescens*	2.7500	
III. Micrococcaceae:		
7. *M. luteus*	0.3646	0.0012
8. *M. conglomeratus*	1.3378	
9. *St. aureus*	1.8825	0.9952
10. *St. albus*	1.8844	
9. *St. aureus*	1.8825	0.0012
11. *St. citreus*	0.4250	
10. *St. albus*	1.8844	0.0012
11. *St. citreus*	0.4250	
12. *Sar. flava*	1.2085	0.0012
13. *Sar. lutea*	0.3875	
IV. Enterobacteraceae:		
14. *A. aerogenes*	2.9322	0.0014
15. *A. cloaceae*	2.3784	
16. *Ser. marcescens*	2.4405	0.0024
17. *Ser. plymouthensis*	2.7848	
18. *Pr. vulgaris*	2.7315	0.0020
19. *Pr. mirabilis*	2.1077	
20. *S. schottmulleri*	2.3865	0.0058
21. *S. paratyphi* A	2.1441	
V. Bacteriaceae:		
22. *F. synxanthum*	1.5015	0.0196
23. *F. acetylicum*	1.7829	
VI. Bacillaceae:		
24. *B. subtilis*	0.0750	.....
25. *B. mesentericus*	No growth	
26. *B. mycoides*	0.2750	0.0014
27. *B. megatherium*	1.3646	

bacterium to support a division rate of *Colpidium* approaching its control, and even in the small difference in mean division rate of 0.1085, P equaled 0.0204. As seen in Figure 2, all other experiments and accompanying controls were quite different, and calculations showed very low P-values, which means that the differences, as observed, had high statistical significance.

Bacteria	Difference between Experimental and Control Means
Al. viscosus (3)	1.9367
P. fluorescens (7)	0.2295
Sar. flava (13)	0.7585
Sar. lutea (14)	0.5317
Ser. marcescens (18)	0.6749
Ser. plymouthensis (19)	0.1085
S. schottmulleri (22)	0.5683
S. paratyphi A. (23)	0.8707

From Figures 1 and 2 certain further relationships may be abstracted. In both figures the broken line represents the average division rate of *C. colpoda* on its respective control bacterium. The average for *P. fluorescens* was 2.7917 ± 0.0534 (standard error) and for *A. aerogenes* was 1.8597 ± 0.1961. These two results should be taken seriously, since the first is based on fourteen separate experiments of four different lines each, and the

TABLE 4

RELATIONSHIPS OF TWO OR MORE BACTERIAL SPECIES OF A GENUS DETERMINED BY THEIR EFFECT ON DIVISION RATE OF *C. colpoda* WHEN TAKEN FROM TWO DIFFERENT BACTERIAL FOODS

Relation Similar	Relation Reversed	Relation Uncertain
Alcaligenes	*Sarcina*	*Staphylococcus aureus* vs. *albus*
Pseudomonas	*Proteus*	*Bacillus subtilis* vs. *mesentericus*
Micrococcus	*Salmonella*	
Staphylococcus aureus vs. *citreus*	*Flavobacterium*	
albus vs. *citreus*		
Aerobacter		
Serratia		
Bacillus megatherium vs. *mycoides*		

second on eighteen such experiments. Using the method described above for testing probabilities, these two averages are significantly different, with a P-value of 0.0000.

Omitting the performances of *Colpidium* on the two species of *Vibrio* and *B. mesentericus* because of its inability to survive for 10 days on these bacteria when taken from *P. fluorescens*, the experimental series (Figs. 1 and 2) were treated statistically, so that the division rates for *C. colpoda* grown on the same species of experimental bacteria in these two series could be compared. For this purpose the two performances of *Colpidium* on each of the twenty-seven bacteria were paired, and Student's method of paired com-

parisons was applied. It was found that, in the cases tested, *Colpidium* taken from *P. fluorescens* and grown on suspensions of different experimental bacteria was significantly different ($P = 0.0000$) from experimental animals taken from *A. aerogenes* cultures. It is interesting to note that this difference was not due to a consistently low or high division rate of animals taken from either of the control bacterial suspensions, for, of these twenty-seven pairs of experiments, animals taken from *P. fluorescens* had higher division rates than those grown on suspensions of *A. aerogenes* for fourteen pairs, while the reverse was true for thirteen pairs when *C. colpoda* was taken from *A. aerogenes*.

With regard to specific differences in a genus of bacteria, in most cases, if *C. colpoda* had a higher division rate on one of two species when taken from *P. fluorescens*, this same relationship held when it was taken from *A. aerogenes*. In a few cases this relationship did not hold. The relationships for all sixty experiments of *C. colpoda* taken from *P. fluorescens* and *A. aerogenes* and grown on the same experimental bacteria are shown in Table 4.

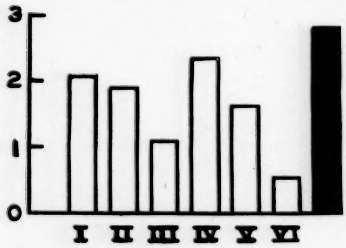


FIG. 4.—The white bars show the average 10-day division rates of *C. colpoda* grown on twenty-seven species of bacteria arranged as family averages. The black bar is the average division rate of the controls for all six families grown on *P. fluorescens*. Ordinates show division rates; abscissae give numerals assigned to families in Table 2. *Vibrio* sp. and *B. mesentericus* not included in averages of *II* and *VI*, respectively (see text).

Finally, it was possible to interpret the data in still another way. In Figure 4 all of the division rates of *Colpidium* grown for 10-day periods on experimental bacteria, as shown in Table 2, were plotted according to families. Since the history of *C. colpoda* grown on *P. fluorescens* was so much more uniform than that for *A. aerogenes*, it is more conservative and accurate to use only the data from the *P. fluorescens*—controlled experiments for this purpose. The available data show that the species in the Enterobacteriaceae supported the highest reproduction rates of *C. colpoda*, and Micrococcaceae and Bacillaceae the lowest division rates. The Rhizobiaceae, Pseudomonadaceae, and Bacteriaceae seem to form an intermediate group, as far as acting as food for *Colpidium* was concerned. Evidently, the Enterobacteriaceae could be said to contain the best food organisms for *C. colpoda*, for, with the exception of the Bacteriaceae, which had only two representatives tested, the Enterobacteriaceae were the only family which had no toxic or decidedly unfavorable species. The Enterobacteriaceae were represented in this work entirely by Gram-negative species, all of which grew very quickly in pour plates. With the exception of the Bacteriaceae, which could not be grown in the usual dilutions in pour plates but supported a relatively high division rate of *Colpidium*, the vibrios, micrococci, sarcinae, and spore-formers all either did not grow or grew poorly in pour plates and also did not support very high productions of *Colpidium*. This appears to be related to the metabolism of the bacteria rather than to their abundance, since there were certainly large numbers of bacterial cells present.

A careful survey of the physiological properties of the above families in *Bergey's Manual of Determinative Bacteriology* (1939) gave no hint as to the reason for one family of bacteria being able to support a higher division rate of *C. colpoda* than another. It is generally known in bacteriology that *Escherichia*, *Aerobacter*, *Salmonella*, *Proteus*, and *Eberthella*, all belonging to the Enterobacteriaceae, have antigenic similarities. This fact

is of interest in connection with the homogeneity of the division rates of *C. colpoda* when grown on species of this family.

Since there are thousands of species of bacteria, only a small fraction of which can be readily obtained, much less grown in the laboratory, it is not likely that more than a small fraction will be tested as food for Protozoa. As mentioned earlier, type species of any genus were used whenever obtainable, so that as representative a sample as possible was tested. Of the thirty-one species of bacteria used, *C. colpoda* was maintained for a 10-day period on twenty-seven of them; therefore, it is safe to conclude that *C. colpoda* may be a polyphagous feeder and approaches euryphagy much more closely than it does stenophagy. Until it can be proved that Protozoa are selective feeders in nature and the synergic relations between bacteria are understood, any definite assignment of certain monoflorae as natural food for particular Protozoa must be withheld.

Hydrogen-ion concentration appeared to have little effect in the present experiments; in the large majority of experimental and control bacterial stock suspensions the pH remained very close to 6.4, while the total range was from 6.0 to 8.0. Only in the cases of the sarcinae, micrococci, and spore-formers were high pH readings taken; therefore, this may, in part, explain the poor showing of *Colpidium* grown on these bacteria. As the pH readings were taken only on the stock bacterial suspensions that were kept in a cold room, it is quite possible that profound pH changes took place in the 24 hours that the Protozoa were in the small 0.2-ml. drops. This may have been due to a germination of spores or to actual growth of the bacteria utilizing their own dead cells or the decomposition products thereof. Such a supposition is suggested by the fact that growth of all of the spore-formers which had encysted in the cold room was observed during the daily 24-hour experimental period.

With the exception of the extreme toxicity of *Ch. violaceum* little difficulty was experienced in maintaining *C. colpoda* on chromogenic bacteria. When *Ch. violaceum* was grown at 25° C., a 1/100 dilution[8] of the concentrated suspension killed the *Colpidium* in less than three-quarters of an hour, while, when the same bacterium was grown at 37° C., much less of the deep-violet pigment was formed, and the Protozoa survived for about 8 hours. The other species in the same genus, *Ch. viscosum*, produced very little pigment and supported a good reproductive rate of Protozoa. Brilliantly pigmented stocks of both *Ser. marcescens* and *P. aeruginosa* were used with good results, although *Colpidium* died in drop cultures of the latter when they were allowed to run longer than 24 hours. The unpigmented strain of *Ser. plymouthensis* also supported one of the highest division rates of all bacteria used. Both species of *Flavobacterium* produced pigment, neither of which seemed to have an especially deleterious effect on *C. colpoda*. In the cases of *P. aeruginosa* and *F. synxanthum*, pigments of similar color were produced; and, although the action of both of these bacteria caused the veal infusion in the Kolle flasks to turn a fluorescent greenish yellow, this color must not have been taken up entirely by the solid medium, for the same color was imparted to the concentrated stock bacterial suspension. However, the stock used for protozoan growth was only roughly a 1/100 dilution of this concentrated stock, so that the pigment was perhaps below the threshold of its deleterious action when used for experimental purposes.

Although no record was kept of the size of individual *C. colpoda* grown on different bacteria, inspections indicated there was little, if any, relation between the dimensions

[8] Colonies of *C. violaceum* did not grow in pour plates even in very low dilutions; for that reason the actual numerical concentration of this stock could not be determined.

of the individual and division rate. Some rapidly dividing cultures had relatively small individuals, while others regularly had forms which were quite large. The spore-formers supported very large colpidia, while *St. citreus* and the *P. fluorescens* controlled experiment of *Sar. lutea* produced very small ciliates with very low division rates in all three cases.

GENERAL DISCUSSION

Since Hetherington (1933, 1934) and Chatton and Chatton (1927*a*, *b*) are the major experimenters to have actually used *C. colpoda* in bacterial-protozoan studies, their work can be cited for comparison with this study. In general, this work is consistent with Hetherington's results, which it greatly extends. Chatton's brief reports on the action of the chromogenic bacteria on *C. colpoda* agrees with neither; however, since these men gave no indication of the actual concentrations of bacteria used, it is difficult to state categorically whether a real conflict exists. In 1934 Hetherington described somewhat vaguely the different effects that washed bacteria had on *C. colpoda*, in contrast with unwashed bacteria. Evidently results obtained in the present work are more comparable to his work with washed bacteria, for no agar substrate was carried into the bacterial suspensions used in these experiments. Pooling the bacteria studied in his 1933 work, which, according to Hetherington's own statement, were of doubtful purity because the *Colpidium* used was not sterile, with a few studied in his 1934 paper, a tabular comparison (Table 5) can be made between his results and those obtained in the present study. As the colpidia whose division rates are shown in the present paper were taken from cultures of *P. fluorescens* and of *A. aerogenes*, their division rates doubtless would differ from those of Hetherington, for in his 1933 work the *Colpidium* was taken from a "favorable medium (malted milk)." For these or other reasons, while certain similarities exist, there are as many differences.

Concerning the above effect of the source of the experimental Protozoa, it might be recalled that *C. colpoda* taken from *P. fluorescens* and grown on the same experimental bacteria as those taken from *A. aerogenes* differed statistically in their division rates. Those results are quite consistent with the host-selection principle of Hopkins (Allee and Park, 1939), which suggests that animals may at times select their habitat as a result of their previous experience. At any rate, with these Protozoa the past history of having lived in one bacterial suspension affected their performance in a suspension of another bacterium.

In 1933 Johnson found that very heavy inocula of *P. fluorescens* often killed the hypotrich *Oxytricha*, while lower concentrations of the bacterium proved to be excellent as a source of food. The use of supraoptimal inoculations of chromogenic bacteria may, in part, help to explain the disagreement between the present work and that of Chatton and Chatton (1927*a*, *b*) on *C. colpoda* and of Kidder and Stuart (1939) using *Colpoda*. All of these workers found *Ser. marcescens*, *P. fluorescens*, and *P. aeruginosa* to have deleterious effects on their experimental animals, while in the present work no such effect was observed. However, this paper, using *C. colpoda*, is in agreement with Kidder and Stuart (1939) on the action of *Ch. violaceum* on *Colpoda*, while the longer survival of *C. colpoda* on the less pigmented strain grown at 37° C. suggests that pigment actually caused the deleterious effect. Both the *Ch. viscosum* and the *Ser. plymouthensis* used in this work were unpigmented and supported a high division rate for *C. colpoda*. These

results are quite in agreement with the general observation that unpigmented strains of pigmented bacteria are better food for Protozoa than the pigmented.

Since Kidder and Stuart (1939) were able to grow *Colpoda* on bacteris suspended in distilled water, and in this work *C. colpoda* was grown on bacteria suspended in artificial pond water as well as in the double distilled water which was used in preliminary experiments, it is surprising that Hetherington (1933) insists that "no bacterium was discovered which, when suspended in fresh water (Peters' medium), would support growth

TABLE 5

TABULAR COMPARISON WITH HETHERINGTON'S DATA

Bacteria Used	Growth or Division Rate of *C. colpoda*		
	Hetherington's Results*	Present Work (Av. of Four Lines for 10 Days)	
		P. fluorescens	*A. aerogenes*
Al. fecalis	Fair	1.54	0.88
P. aeruginosa	Fair	1.07	1.84
P. fluorescens	Large	2.75	3.00
M. conglomeratus	1.99	1.34	0.73
E. coli	Toxic	2.14	1.22
A. aerogenes	4.33	2.93	1.82
	2.74	.....	.....
	Very large	.....	.....
A. cloacae	Fair	2.38	1.51
Pr. vulgaris	Large	2.73	1.25
B. subtilis	Large	0.08	0.33
B. mesentericus (unwashed)	2.70	Survived 1 day	0.20

* Verbal descriptions of growth were taken from Hetherington's 1933 paper, in which the length of the experiments was not mentioned; numerical descriptions from his 1934 paper indicate 7-day averages.

of *C. colpoda* for more than a few generations." As mentioned earlier (see "Material and Methods"), stock protozoan cultures were kept regularly for 1 month. At widely fluctuating room temperatures old stocks were kept for 3 months, and at 25°.5 ± 0°.5 C. they ran for 6 months, while in a cold room at 6° C., *C. colpoda* in an *A. aerogenes* suspension has been maintained for 10 months. It must, therefore, be concluded either that the methods used in this work and by Kidder and Stuart (1939) were imperfect or that Hetherington has not used optimal inocula of food bacteria or that his preparation of Peters' medium was somewhat toxic.

There are certain strong suggestions (Table 3) that different species of bacteria be-

longing to the same genus may be quite different in their ability to support protozoan growth. It is too early to state definitely that these differences were any more than tendencies; but, when it is also seen that Cleveland (1928), Hetherington (1934), Kidder and Stuart (1939), and Leslie (1940), using widely diverse species of Protozoa, all found indications that strains of the same bacterial species had different growth-promoting effects when used as food for the several experimental animals, it is not surprising that similar differences may exist among species of bacteria in the same genus.

The science of bacteriology has solved many of its technical problems in such a way that bacteriologists all over the world can make use of the same techniques and, therefore, obtain comparable results. Many of the techniques used in bacteriology can be carried over into the study of Protozoa that will live on nonliving-particulate or nonparticulate media. However, under present-day culture conditions at least, many holozoic Protozoa must have living food; for that reason the old problem of method again arises. Usually, bacterial cultures are seeded with large inocula; and, because of the rapid division rate of the bacteria, the standard length for which they are allowed to grow is generally 24 hours. The same techniques will not give comparable results with Protozoa grown on different bacteria unless a gross inoculum of Protozoa in mass cultures is used, for it is usually impossible in 24 hours to obtain enough animals for a sampling count if more than a few cubic centimeters of bacterial suspension are used. Small samples taken over a period of time from mass cultures do give valuable information about the shape and amplitude of the growth curve of numbers in the growing population; but, unless the same-sized inoculum, the same amount of food, the same volume, and the same time lapse are used, very different results will be obtained (Hall, 1941*a*). Inconsistent use of mass culturing as a technique has led to the present somewhat confused state of experimental work in this field.

Unfortunately, the growth of Protozoa in isolation cultures using bacteriological methods plus daily counts of Protozoa is highly laborious, especially when bacteria-free protozoans are grown on a monoflora. However, for the reasons cited earlier, it has obvious advantages over the mass-culture technique for establishing simple accurate relationships between Protozoa and species of bacteria. The erratic results obtained by Hetherington (1934) for *C. colpoda* grown on *A. aerogenes* in mass culture and the same type of results obtained in isolation culture for the same organisms in this paper indicate that, for some organisms at least, many readings must be made to obtain merely a comprehensive description of existing relationships. These extra readings are needed regardless of whether mass or isolation cultures are to be used. Present experience indicates that fifteen to twenty entirely separate determinations should suffice.

Protozoa growing in mass cultures may approach closely the condition in nature, while growing Protozoa in isolation cultures is an artificial method which can be justified since it allows the analysis of many of the relations found in the larger complex. Mass cultures produce so many individuals that statistics are quite applicable; but much significance is lost, for only surviving individuals are used to describe what is happening within the population. On the other hand, many readings using isolation cultures are necessary for adequate tests of statistical significance. However, so much more accuracy is gained by the second method that for certain types of study the extra effort is warranted.

Several authors (Cleveland, 1928; Hetherington, 1934; Kidder and Stuart, 1939; Leslie, 1940) have pointed out that different strains of bacteria of the same species affect the division rates of Protozoa in different ways. For a study of such happenings the isolation-culture method is useful, because the 24-hour counts allow the interaction be-

tween bacteria and Protozoa to be studied in much the same way that other physiological tests are used in the study of bacteria. It is quite possible that subtle differences in different strains of bacterial species may be explored by growing Protozoa on them, especially if relatively standardized and stable Protozoa can be found. In this case, Protozoa would become indicators for the use of the bacteriologist; but care must be taken, for Hetherington (1934) found that Protozoa react quite differently to bacteria grown in bacterial broths, in contrast with those suspended in balanced salt solutions. The increase in the division rate found in his work when *Colpidium* was grown in bacterial suspensions in broths may have been due to the partial utilization of the broth by the protozoan itself or to the added food supplied by an increased number of bacteria growing in the broth.

It is commonly known that certain domesticated herbivores cannot be successfully raised on poor fodder containing weeds of little nutritional value. As Allee (1941) has pointed out, the early investigators in protozoölogy may have inadvertently included weeds in their "hay-tea" cultures. Possibly such weeds might have affected the Protozoa grown in hay-tea; however, the present analysis has gone a step further, for now a less obscure interaction between Protozoa and their food has been established. Certain bacteria, because of their excellency as food, might be called "microfodder," while others, of low nutritional value, are nothing more than weeds, as far as the polyphagous "microherbivore" *C. colpoda* is concerned.

SUMMARY

1. A controlled isolation-culture technique was used to study the bacterial-protozoan relationships between thirty-one species of Eubacteriales and *C. colpoda*.

2. In all but a few cases the experimental bacteria caused significant differences in the division rate of *Colpidium*, as compared with simultaneous tests of its division rate on control bacteria.

3. There are indications that *C. colpoda* is a polyphagous bacterial feeder, since it was maintained for a 10-day period in suspensions of twenty-seven out of thirty-one bacteria used.

4. *Colpidium* grown on *P. fluorescens* differed significantly in division rate from that grown on *A. aerogenes*.

5. The source of experimental animals was important, for *Colpidium* taken from *P. fluorescens* and from *A. aerogenes* and placed on suspensions of the same experimental bacteria react significantly differently as to division rates and survival time.

6. The family Enterobacteriaceae supported the highest division rate of *C. colpoda* out of six families, while the Bacillaceae supported the lowest.

7. It is not yet apparent what physiological property or properties of these Gram-negative organisms common to the whole family Enterobacteriaceae makes its members especially good food organisms for *C. colpoda*.

8. From preliminary statistical tests it appears that different species of bacteria belonging to the same genus have significantly different growth-promoting powers when used as food for *C. colpoda*.

LITERATURE CITED

ALLEE, W. C. 1941. Integration of problems concerning protozoan populations with those of general biology. Amer. Nat., **75**:473.

ALLEE, W. C.; BOWEN, E. S.; WELTY, J. C.; and OESTING, R. 1934. The effect of homotypic conditioning of water on the growth of fishes and chemical studies of the factors involved. Jour. Exper. Zoöl., **68**:183.

ALLEE, W. C., and PARK T. 1939. Concerning ecological principles. Science, **89**:166.
BERGEY, D. H.; BREED, R. S.; MURRAY, E. G. D.; and HITCHENS, A. P. 1939. Bergey's manual of determinative bacteriology. Baltimore: Williams & Wilkins Co. Pp. 1032.
BUCHSBAUM, R., and LOOSLI, C. G. 1936. Methods of tissue culture in vitro. Chicago: University Press. Pp. 81.
BUTTERFIELD, C. T.; PURDY, W. C.; and THERIAULT, E. J. 1931. Experimental studies of natural purification in polluted water. IV. The influence of plankton in the biochemical oxidation of organic matter. U.S. Pub. Health Repts., **46**:393.
CALKINS, G. N. 1933. The biology of the Protozoa. Philadelphia: Lea & Febiger. Pp. 607.
CALKINS, G. N., and SUMMERS, F. M. 1941. Protozoa in biological research. New York: Columbia University Press. Pp. 1148.
CHATTON, E., and CHATTON, M. 1927*a*. Sur le pouvoir cytolysine immédiat des cultures de quelques Bactéries chromogenes. Comp. rend. Soc. biol., **97**:289.
———. 1927*b*. Sur les propriétés et la nature de la cytolysine du Bacille pyocyanique. Differenciation d'avec les pigments, les ferments, et les protides. *Ibid.*, **97**:292.
CLEVELAND, L. R. 1928. The suitability of various bacteria, molds, yeasts, and spirochaetes as food for the flagellate *Trichomonas fecalis* of man as brought out by the measurement of its fission rate, population density, and longevity in pure cultures of these microorganisms. Amer. Jour. Hyg., **8**:990.
CUTLER, D. W., and CRUMP, L. M. 1923. The rate of reproduction in artificial culture of *Colpodium colpoda*, Part I. Biochem. Jour. **17**:174.
FISHER, R. A. 1925. Applications of "Student's" distribution. Metron, **5**: No. 3, p. 90.
HALL, R. P. 1941*a*. Food requirements and other factors influencing growth of Protozoa in pure cultures, in Protozoa in biological research, ed. G. N. Calkins and F. M. Summers (New York: Columbia University Press, 1941).
———. 1941*b*. Populations of plant-like flagellates. Amer. Nat., **75**:419.
HAMMOND, E. C. 1938. Biological effects of population density in lower organisms. Quart. Rev. Biol., **13**:421; **14**:35.
HETHERINGTON, A. 1933. The culture of some holotrichous ciliates. Arch. f. Protistenk., **80**:255.
———. 1934. The role of bacteria in the growth of *Colpidium colpoda*. Physiol. Zoöl., **7**:618.
JAHN, T. L. 1934. Problems of population growth in Protozoa. Cold Spring Harbor Symp. Quant. Biol., **2**:167.
JOHNSON, W. H. 1933. Effects of population density on the rate of reproduction in *Oxytricha*. Physiol. Zoöl., **6**:22.
———. 1941. Populations of ciliates. Amer. Nat., **75**:438.
KIDDER, G. W., and DILLER, W. F. 1934. Observations on the binary fission of four species of common free-living ciliates, with special reference to the macronuclear chromatin. Biol. Bull., **67**:201.
KIDDER, G. W., and STUART, C. A. 1939. Growth studies on ciliates. I. The role of bacteria in the growth and reproduction of *Colpoda*. Physiol. Zoöl., **12**:329.
LESLIE, D. 1940. Nutritional studies of *Paramecium multimicronucleata*. I. Quantitative and qualitative standardization of the food organisms. Physiol. Zoöl.,**13**:243.
PARPART, A. K. 1928. The bacteriological sterilization of *Paramecium*. Biol. Bull., **55**:113.
PETERS, R. A. 1921. The substances needed for the growth of a pure culture of *C. colpoda*. Jour. Physiol., **55**:1.
PHELPS, A. 1935. Growth of Protozoa in pure culture. I. Effect upon the growth curve of age of inoculum and amount of the inoculum. Jour. Exper. Zoöl., **70**:109.
RICHARDS, O. W. 1941. The growth of the Protozoa, in Protozoa in biological research, ed. G. N. Calkins and F. M. Summers (New York: Columbia University Press, 1941).
SANDON, H. 1927. The composition and distribution of the protozoan fauna of the soil. Edinburgh: Oliver & Boyd. Pp. 237.
"STUDENT." 1925. New tables for testing significance of observation. Metron, **5**: No. 3, p. 105.
TAYLOR, C. V., and WAGTENDONK, W. J. VAN. 1941. Growth studies of *Colpoda duodenaria* in the absence of other living organisms. Jour. Cell. and Comp. Physiol., **17**:349.

THE PRESENCE OF CHLORIDE IN MUSCLE FIBERS[1]

L. V. HEILBRUNN AND PAULINE G. HAMILTON
Zoölogical Laboratory, University of Pennsylvania

IN A famous paper published in 1902 Overton concluded that the loss of irritability of frog muscles immersed in sugar solutions was due to a diffusion from the muscles of sodium chloride, and he was led to assume that the sodium chloride was present not in the muscle fibers but in the interstitial spaces between the fibers. Indeed, he stated that if one knew the concentration of chloride in a given muscle, one could calculate the volume of interstitial space on the basis of the assumption that all the chloride was in this space at a concentration equal to that present in the blood plasma. Ably championed by Peters (1935) and by Fenn (1936), this idea of Overton's has gained general acceptance. After considering the indirect evidence of various authors, Peters (p. 133) states: "Even in the absence of all this evidence one would almost be compelled to believe that muscle cells were practically devoid of chloride simply because the large quantities of protein and phosphate in addition to bicarbonate and, at times, lactic acid, are sufficient to neutralize all the base which they contain, leaving none to combine with chloride." If muscle cells contain no chlorine, it is only logical to assume that other types of cells are also lacking in this element. There has been no lack of support for this view. Thus Truax (1939) presented evidence to show that all the chloride in liver tissue was in the spaces between the cells, and other authors—e.g., Harrison, Darrow, and Yannet (1936) and Bourdillon (1937)—have extended the doctrine to all types of tissue cells. Such an exaggerated view has not met with the support of more cautious workers (see, e.g., Manery and Bale, 1941, and references cited by them). Nevertheless, many clinical investigators have adopted the theory and have used chloride analyses of tissues and organs as a convenient and accurate method of determining the volume of extracellular space. Indeed, the term "chloride space," regarded as a synonym of "extracellular space," is well established in the fields of physiology, biochemistry, and medicine. The literature on the subject is voluminous.

However, not all authors have been content to regard the muscle cell as totally devoid of chloride. Conway, Kane, Boyle, and O'Reilly (1939), after studying the diffusion of chloride from the excised sartorius muscle of the frog, concluded that some of the chloride which diffused out was originally contained within the fibers. During the past few years the diffusion of chloride into and out of excised muscles has been reported in several other papers: Fisher and Subrahmanyan (1939); Boyle, Conway, Kane, and O'Reilly (1941); Boyle and Conway (1941). In such excised muscles, chloride seems to diffuse into and out of muscle fibers. However, these results are all for muscles outside of the body; and it is quite proper to maintain, as does Dean (1941), that *in vivo* no such diffusion occurs. Indeed, Fisher and Subrahmanyan wonder what factor "normally operative in the organism to maintain chloride impermeability of the muscle cells is diluted or destroyed during soaking in Ringer's solution."

Almost all the arguments in favor of the view that the chloride of muscles is contained

[1] This research has been supported by a grant from the Rockefeller Foundation.

between the cells and not in them are based on indirect evidence. There has been very little attempt at a direct analysis of muscle cells. In 1938 Gersh endeavored to show by histochemical tests that the cells were chloride-free. This work has been harshly but justly criticized by Scott and Packer (1939). As a matter of fact, it is hard to understand how Gersh's work could ever have been taken very seriously, for he tested the muscle cells only after they had been frozen in liquid air, dried in a vacuum, infiltrated with liquid paraffin at 60° C., and cut into sections which were so thin that they were only about one-sixth the diameter of a muscle fiber in thickness. During such treatment chloride might have passed in either direction across the membranes or the fragments of the dead cells. In 1941 Dean studied the chloride content of carefully prepared isolated muscle fibers. Each individual fiber was tested separately, after being dipped for less than a second in a solution containing 9 parts of isotonic mannitol and 1 part of isotonic sodium nitrate. The fibers had previously been dissected in chloride-containing Ringer's solution. On analysis Dean found that the fibers contained 15.5 micromoles of chloride per cubic centimeter ± 35 per cent, but he attributed this chloride to the previous contact with Ringer's fluid, and he inclined to the opinion that the chloride was only at the surface and not really within the fiber. In general, fibers with larger diameter showed relatively less chloride—a fact consistent with the view that the chloride is on the surface. Moreover, horsehair with a surface film of Ringer's solution, after being washed in the mannitol–sodium nitrate solution in the same way as the fibers, still showed chloride, although only about a third as much as the fibers. The lesser amount of chloride on the horsehair Dean thought might "be partially explained by the fact that the horsehair was stiff and moved faster through the washing solution. It also seemed to have less Ringer's solution on its surface than the fibers."

Dean's results do not prove conclusively either that the frog muscle fiber is chloride-free or that it contains chloride, and in his summary he merely states that "there were indications that most of the chloride was on the surface of the fiber." It is only natural, therefore, that workers in the field should continue to regard all muscle chloride as extracellular and to base their experiments on this assumption (cf. Eichelberger and McLean, 1942).

If the question is to be settled, further work is necessary. Obviously, if the fibers Dean analyzed had been dissected in a chloride-free solution instead of in Ringer's solution, there could have been no question of a wetting film of liquid containing chloride. In our experiments all analyses were performed on fibers dissected from the muscle in a chloride-free solution.

METHODS

Isolated fibers were in all cases obtained from the adductor magnus muscle of *Rana pipiens*. This muscle has long parallel fibers, and it lacks the tough connective-tissue sheath found in many other muscles. Before dissection the muscle was placed in a chloride-free Ringer's solution (5.4 gm. $NaNO_3$, 0.13 gm. KNO_3, and 0.15 gm. $Ca[NO_3]_2$ per liter, buffered to pH 7.5 with $m/15$ Na_2HPO_4). No attempt was made to dissect out fibers for their entire length. Such dissection is very time consuming, and it offers little advantage for our experiments. As a matter of fact, there is a real disadvantage, for it would not have been wise for us to leave the fibers for a long time in the nitrate Ringer's fluid. Accordingly, each fiber was cut at both ends, and the length of the cut piece was measured with a millimeter rule. Following the measurement of its length, the fiber was washed

twice in the chloride-free Ringer's fluid and was then sucked up with an ordinary medicine dropper and transferred to a slide under a microscope, so that its diameter could be measured with the aid of a micrometer eyepiece. Not infrequently the diameter of the fiber varied along the length of the piece. In such instances a position of approximately average thickness was chosen from the measurement. With such methods no great accuracy could be obtained; but it was necessary to work rapidly, and it was assumed that inaccuracies in the measurement of fiber diameter would in general cancel out. In calculating the percentages of chloride in the fibers, we took the specific gravity of the fibers as 1.05. The specific gravity of frog muscle (by actual test) is approximately 1.05, and the specific gravity of the fibers can scarcely be significantly greater than this. In dissecting isolated fibers, strips of blood-capillary, connective-tissue, or nerve fiber are frequently dissected out along with the muscle fiber. In those instances in which this extraneous material was present in any great amount the fiber was discarded. After a fiber had been washed and measured, it was picked up with a dissecting needle and transferred to a small vial resting on a cake of dry ice. After the fibers had been thoroughly frozen, they were ground up with a glass rod, and then a small quantity of hot water was added to them. The vial was then sealed and placed on a steam bath for a half-hour. After the water had cooled, it was tested for chloride by Wigglesworth's micromethod (1937). In other experiments fibers, after being frozen and ground up, were digested with nitric acid in accordance with the standard Van Slyke method (1923). In a few cases the Wilson and Ball (1928) modification of the Van Slyke method was employed. In either case the Wigglesworth micromethod was used in the titration. This is essentially a micro-Volhard-method. In all the experiments the reagents were tested and found to be chloride-free.

Small pieces of muscle were extracted and tested in the same manner as the fibers. In some experiments larger pieces of muscle were analyzed by macromethods; that is to say, ordinary burettes were used instead of microburettes.

Histochemical tests on isolated fibers were performed with one of the two reagents recommended by Gersh (1938), his reagent 1. This consists of a 60 per cent solution of silver nitrate diluted with a sufficient quantity of concentrated H_3PO_4 to prevent the precipitation of rather large concentrations of phosphates and then saturated with silver chloride; after filtering, two or three drops of water are added to each 10 cc. of reagent. In other tests the reagent was made up with 60 per cent HNO_3 instead of H_3PO_4. The substitution of nitric acid for phosphoric acid is believed wise, for Macallum (1905) had thought that the presence of creatine in muscle might interfere with the test because of its reducing action. Such reduction would be impossible in the presence of strong nitric acid. The technique used for the detection of chloride is that of Macallum. In this method AgCl, formed by reaction of chloride with the reagent, is reduced either by direct sunlight or by the use of a mercury-arc lamp. A positive test is indicated by the appearance of yellowish-brown or black masses.

RESULTS

Analyses of the water extract of fibers dissected in chloride-free Ringer's fluid and then washed in this fluid showed that the fibers themselves had an average chloride content of 41.1 milliequivalents per kilogram of muscle fibers. The results of the individual tests are shown in Table 1. The chloride content of small pieces of muscle treated in the same way as the fibers (except for the washings in chloride-free Ringer's solution) was found

to average 51.5 milliequivalents of chlorine per kilogram of muscle (see Table 2). Accordingly, we can conclude from our data that the muscle fibers contain 80 per cent as much chloride as does the whole muscle.

In view of the fact that this conclusion is strongly opposed to existing beliefs, we were led to examine our findings as critically as possible. In the first place it should be noted that in analyzing water extracts of muscle we are using an unorthodox method. It is true that Krogh (1937) also used water extracts of frog muscle for analysis; but in general the almost universal method of studying tissue chloride is to subject the tissue to strong nitric acid before beginning the analysis. Accordingly, we determined to repeat our experiments with the Van Slyke method (see Van Slyke, 1923), digesting the muscle in the

TABLE 1

THE CHLORIDE CONTENT OF ISOLATED FROG MUSCLE FIBERS AS DETERMINED FROM THE WATER EXTRACT

Experiment Number	Number of Fibers	Total Volume of Isolated Fibers (In Mm.^{3})	Total Volume of Extract (In Ml.)	Mg. Cl in Extract	Percentage of Cl in Fibers	Milli-equivalents of Cl per Kilogram of Fibers
1	65	9.1481	0.1	0.01536	0.160	45.1
2	81	9.2745	0.1	0.01390	0.143	40.3
3	76	7.7597	0.25	0.01210	0.149	42.0
4	72	9.8476	0.15	0.01418	0.137	38.6
5	72	9.9308	0.15	0.01549	0.149	42.0
6	76	13.8928	0.10	0.01910	0.131	36.9
7	76	11.9236	0.10	0.02158	0.172	48.5
8	74	13.8518	0.30	0.02080	0.143	40.3
9	72	14.7927	0.20	0.02130	0.137	38.6
10	75	15.040	0.20	0.02260	0.143	40.3
11	75	11.6158	0.20	0.01750	0.143	40.3
12	74	16.8850	0.15	0.02431	0.137	38.6
13	73	24.3995	0.10	0.03805	0.148	41.7
14	75	16.3020	0.10	0.02740	0.160	45.1
15	75	14.9069	0.10	0.02147	0.137	38.7

Average: 41.1 milliequivalents per kilogram of muscle fibers.

presence of nitric acid plus silver nitrate. The results of this second series of experiments are shown in Tables 3 and 4. As before, the fibers were dissected, washed, and measured in chloride-free Ringer's fiuid, and they were then digested and their chloride content determined. With this technique it was found that the fibers contained 24.0 milliequivalents of chlorine per kilogram of fibers. Pieces of muscle analyzed in the same way contained 30.8 milliequivalents of chlorine per kilogram of muscle. Thus with this standard technique we were able to show that the muscle fibers contain 78 per cent as much chloride as does the whole muscle.

In his analyses of single isolated fibers Dean (1941) used the same technique as we did in this second series of experiments. Our results for isolated fibers are somewhat higher than his, for we found an average of 24.0 milliequivalents of chlorine per kilogram, as compared with Dean's average of 15.5 milliequivalents. However, Dean's results are somewhat more variable than ours, and in two of his eight determinations, he obtained values of approximately 25 milliequivalents per kilogram.

It is clear that, whether we analyze the water extract or the nitric acid digest of the fibers, we are able to discover a goodly amount of chloride in them. By either technique we can show a high per cent of the chloride to be intracellular rather than extracellular. This is our major contention. However, the wide discrepancy between the values obtained for the water extracts and those obtained for the nitric acid digests led us to inquire as to which of the two series of values is the more nearly correct.

An obvious criticism of the water-extract technique is the fact that such extracts might contain substances such as proteins and that these proteins would interfere with the Volhard silver titration, which forms the basis of the Wigglesworth method. Inasmuch as the extraction was done over a steam water bath, it is not very likely that much protein

TABLE 2

THE CHLORIDE CONTENT OF SMALL PIECES OF FROG MUSCLE AS DETERMINED FROM THE WATER EXTRACT

Experiment Number	Weight of Muscle (In Gm.)	Volume of Extract (In Ml.)	Mg. Cl in Extract	Percentage of Cl in Muscle	Milliequivalents of Cl per Kilogram of Muscle
1	0.16	0.8	0.3472	0.217	61.3
2	0.28	1.2	0.5236	0.187	52.7
3	0.126	0.5	0.2205	0.175	49.3
4	0.140	1.2	0.2548	0.182	51.3
5	0.125	0.7	0.2368	0.185	52.1
6	0.115	0.6	0.1978	0.172	48.5
7	0.123	1.05	0.2088	0.169	47.8
8	0.105	1.0	0.1646	0.156	44.2
9	0.107	1.2	0.1816	0.169	47.8
10	0.128	0.7	0.2701	0.211	59.5
11	0.155	0.9	0.2632	0.169	47.8
12	0.153	1.1	0.3044	0.199	56.1

Average: 51.5 milliequivalents per kilogram of muscle.

would be present in the extracts. Nevertheless, it was thought desirable to test the matter further. Accordingly, small pieces of muscle were extracted with hot water in the usual manner, and the extracts were then divided into two equal portions. One-half of the extract was tested directly with the Wigglesworth microtechnique. The other half of the extract was first digested with nitric acid in the presence of silver nitrate, according to Van Slyke's procedure, and the resulting digest was then analyzed as before. The results of these tests are given in Table 5. In all, six experiments were tried. With the exception of one of them, the two sets of tests gave results in close agreement with one another—remarkably close in view of the fact that the Wigglesworth microtechnique is not extremely accurate. The average of all six experiments showed a difference of only 4.5 per cent between the two sets of tests, and this difference is in favor of the samples subjected to nitric acid digestion. Hence nitric acid digestion of our water extracts is of no help in making the chloride values for these extracts lower and more consistent with the usual values obtained when muscle is digested immediately. In other words, if the Van Slyke method is correct (and our water method wrong), then the Van Slyke technique as

applied to our water extracts should give lower values than those obtained from the water extracts directly.

Accordingly, we were led to suspect the value of the Van Slyke technique as applied to the determination of the chloride content of muscle. In his original paper Van Slyke (1923) stated that "the accuracy of the method with tissues has not been demonstrated

TABLE 3

THE CHLORIDE CONTENT OF ISOLATED MUSCLE FIBERS AS DETERMINED BY THE VAN SLYKE METHOD

Experiment Number	Number of Fibers	Total Volume of Isolated Fibers (In Mm.^{3})	Total Volume of Extract (In Ml).	Mg. Cl in Extract	Percentage of Cl in Fibers	Milli-equivalents of Cl per Kilogram of Fibers
1	74	26.469	0.15	0.028586	0.103	29.0
2	70	17.745	0.05	0.015970	0.086	24.2
3	74	31.023	0.09	0.021095	0.065	18.3
4	68	20.604	0.06	0.017101	0.079	22.3
5	69	16.3468	0.07	0.018635	0.109	30.7
6	68	14.2147	0.015	0.012224	0.082	23.1
7	66	15.435	0.04	0.011730	0.072	20.3

Average: 24.0 milliequivalents per kilogram of muscle fibers.

TABLE 4

THE CHLORIDE CONTENT OF SMALL PIECES OF FROG MUSCLE AS DETERMINED BY THE VAN SLYKE METHOD

Experiment Number	Weight of Muscle (In Gm.)	Total Volume of Extract (In Ml.)	Mg. Cl in Extract	Percentage of Cl in Muscle	Milliequiva-lents of Cl per Kilo-gram of Muscle
1	0.145	2.0	0.16675	0.115	32.0
2	0.293	0.2	0.38969	0.133	36.0
3	0.141	0.15	0.14523	0.103	29.0
4	0.231	0.2	0.24717	0.107	30.3
5	0.0955	0.1	0.11713	0.1218	34.3
6	0.128	0.2	0.13594	0.1062	29.9
7	0.1685	0.2	0.16345	0.097	27.3
8	0.108	0.4	0.13608	0.126	35.5
9	0.167	0.1	0.13025	0.078	22.6

Average: 30.8 milliequivalents per kilogram of muscle.

as completely as with blood, by removing the tissue chlorides and restoring known amounts." In the following experiments small pieces of muscle were placed in an isotonic sucrose solution (0.25 molar) for $1\frac{1}{2}$–5 hours and were then washed for 15 minutes in chloride-free nitrate Ringer's solution. After such treatment, analysis with the Van Slyke method showed complete absence of chloride. The individual pieces of muscle were then each placed in contact with 0.2 cc. 0.1 molar sodium chloride and were left in contact with this solution for times which varied from $1\frac{1}{2}$ to 4 hours. They were then frozen

with dry ice, ground up, and extracted with nitric acid containing silver nitrate, in accordance with the usual Van Slyke technique. Chloride was determined by the Wigglesworth microtitration method (see Table 6).

The results are certainly surprising. A considerable fraction of the chloride added to the chloride-free muscle was not recovered in the titration. Apparently, some constituent of the muscle is capable of binding chloride, and this chloride is not released even after treatment with strong nitric acid.

TABLE 5

ANALYSIS OF WATER EXTRACTS OF MUSCLE WITH AND WITHOUT ACID TREATMENT

Experiment Number	Weight of Muscle (In Gm.)	Total Volume of Extract (In Ml.)	Mg. Cl in Unacidified Extract	Mg. Cl in Acidified Extract (Van Slyke)	Percentage of Difference
1	0.282	1.0	0.40044	0.38352	− 4.2
2	0.173	0.8	0.30621	0.38579	+26.0
3	0.132	0.8	0.27852	0.28644	+ 2.8
4	0.246	1.0	0.45756	0.44526	− 2.7
5	0.260	1.0	0.4524	0.4862	+ 7.5
6	0.336	1.0	0.5308	0.5174	− 2.5

Average percentage of difference: +4.5 in favor of the extract analyzed by the Van Slyke method.

TABLE 6

TEST OF VAN SLYKE'S METHOD WITH KNOWN QUANTITIES OF CHLORIDE TO WHICH CHLORIDE-FREE MUSCLE WAS ADDED

(Muscle freed from chloride by washing in sugar solution)

Test	Weight of Muscle (In Gm.)	NaCl Added (In Mg.)	NaCl Recovered (In Mg.)	Loss of NaCl (In Mg.)	Percentage of Loss
1	0.315	1.152	0.858	0.294	25.5
2	0.168	1.152	0.874	0.278	24.1
3	0.305	1.152	0.906	0.246	21.3
4	0.328	0.876	0.6196	0.2564	29.2
5	0.145	0.876	0.3294	0.5466	62.4
6	0.370	1.152	0.9439	0.2081	18.0

Microtitrations can never be so accurate as titrations made with larger volumes of fluid. Accordingly, it was decided to repeat our test of the Van Slyke method with ordinary burettes. Relatively large pieces of muscle were washed with hot distilled water for periods of 1–6 hours (see Table 7). The flasks containing the pieces of muscle were kept on a steam bath during the period of washing, and the wash water was changed from time to time. After the muscle pieces had cooled, a measured amount of 0.2 molar sodium chloride was added to them. In all but one experiment, 10 cc. of solution were added; in experiment No. 4, only 5 cc. were added. Following a rather long interval, the muscle with its supernatant chloride solution was subjected to the Van Slyke technique, and the

chloride was then determined by an ordinary Volhard titration. In experiments No. 4 and 5, the Wilson and Ball (1928) modification of the Van Slyke technique was used. This consists in adding silver nitrate before the nitric acid, instead of adding these two reagents together.

The results shown in Table 7 are thoroughly consistent. One value—that found in experiment No. 8—is somewhat out of line with the others. This is to be explained by the fact that the washing in this case was for only a single hour, and presumably not all the chloride was removed. As a matter of fact, it is quite possible or even probable that in all the experiments some chloride remained after washing.

In comparing the results shown in Table 6 with those shown in Table 7, it is to be noted that there is a much greater percentage loss of chloride in Table 6. This is partly to be explained by the difference in the conditions of the two series of experiments. In

TABLE 7

TEST OF VAN SLYKE'S METHOD WITH KNOWN QUANTITIES OF CHLORIDE TO WHICH CHLORIDE-FREE MUSCLE WAS ADDED

(Muscle freed from chloride by washing in distilled water)

Experiment Number	Weight of Muscle (In Gm.)	Washed (Hours)	NaCl Added (In Gm.)	Hours in Contact with NaCl	NaCl Recovered (In Gm.)	NaCl Lost (In Gm.)	Percentage of Loss
1	8.7	6	0.11695	16½	0.100	0.01695	14.5
2	6.13	6	0.11695	16½	0.10396	0.0130	11.1
3	4.93	6	0.11695	16½	0.10242	0.0145	12.4
4	4.81	5	0.0589	16	0.05121	0.00769	13.0
5	7.32	5	0.11695	16	0.10292	0.01403	11.9
6	7.0	2	0.1190	2	0.1050	0.0140	11.7
7	7.0	2	0.1190	2	0.10418	0.01482	12.4
8	8.0	1	0.1190	1	0.11324	0.00576	4.8
9	5.0	3	0.1190	2	0.1092	0.0098	8.2
10	5.0	3	0.1190	3	0.1083	0.0107	9.0

the first of the two series the amount of chloride added was relatively small in comparison with the weight of muscle. Actually, gram for gram, the muscle pieces in the second series of experiments accounted for more chlorine than did the pieces in the first series. Thus, if one calculates the chlorine lost per gram of muscle present, the data of Table 6 show an average loss of 0.68 mg. of chlorine per gram of muscle, whereas the data of Table 7 show an average loss of 1.14 mg. per gram of muscle. This greater chlorine loss is correlated with the fact that in the later series of experiments a 0.2-molar sodium chloride solution was used instead of the 0.1-molar solution used in the earlier series. Another series of experiments was performed in which relatively large pieces of muscle, after being washed, were left in contact with 0.1-molar sodium chloride and then tested for chloride by the Van Slyke method. With larger pieces of muscle (or even with smaller ones) it is doubtful whether washing with hot water ever makes the muscle wholly chloride-free. This would introduce a greater error when more dilute solutions were added, and our experiments with the larger pieces in 0.1 normal sodium chloride did not give so consistent or so significant results as those shown in Table 7.

We have been able to show, both by the analysis of hot-water extracts and also by analyses according to the Van Slyke technique, that isolated frog muscle fibers contain considerable amounts of chloride. This chloride is certainly not present as a wetting film on the surfaces of the fibers, but there is, of course, a possibility that it may be chemically combined in the surface. In order to test this point we made some histochemical tests of isolated fibers. The histochemical detection of chloride was carefully studied by Macallum (1905). His method has been sharply criticized by Lison (1936), but we do not believe that Lison's arguments are well founded. Following Lison's criticism, Gersh (1938) suggested a new reagent consisting of 60 per cent silver nitrate but differing from Macallum's reagent chiefly in the fact that it was acidified with phosphoric instead of nitric, acid. According to Macallum, vertebrate muscle fibers are not very favorable objects for histochemical study, for they contain creatine, which can act as a reducing agent. Because of this fact we prepared a reagent which contained, in addition to the silver nitrate, a high concentration of nitric acid (see earlier discussion on methods). In view of the fact that nitric acid is a powerful oxidizing agent, one could scarcely expect any ordinary reduction to occur in its presence.

Both with Gersh's reagent and with our own we were able to demonstrate the presence of chloride within isolated muscle fibers. The tests are not always successful, but a much higher percentage of success can be obtained if the protoplasm of the fiber is first heated or crushed. These injuries seem to release chloride, and this is additional evidence that the chloride within the fiber is, to some extent at least, bound chemically. These histochemical studies will probably be reported more fully in a later paper.

DISCUSSION

It is an interesting and long-known fact that the various tissues and organs of vertebrate animals are not so rich in chloride as the fluids in which they are bathed. This has led to a general belief that the tissue cells are completely, or almost completely, devoid of chloride. Our results do not support this belief. Moreover, we have strong indication that the chloride content of muscle tissue is greater than is commonly supposed and that the ordinary methods used for determining tissue chloride may be in error—at least for muscle.

If it is true that the nitric acid treatment of muscle tissue does not produce digests which contain the original chloride of the muscle in a form in which it can be detected by ordinary methods of analysis, then it may be necessary to revise all the existing estimates of the chloride content of muscle. Moreover, in tissues other than muscle, the acid digestion method may also be unsatisfactory. In general, in the hands of various investigators the acid digestion method has given rather more variable results for tissues than it gives for blood. This in itself should throw suspicion on the method as applied to tissues.

It should perhaps be noted that Krogh (1937) determined the chloride content of tissues and organs of the frog by analyzing the chloride present in water extracts. However, instead of obtaining high values for chloride, Krogh states that his values were slightly below those obtained following complete destruction of the tissue. The reason why Krogh's values for muscle are lower than ours is fairly clear from the description he gives of his method. "To a weighed sample of tissue a suitable amount of acidulated water was added, the whole brought to boiling and left for extraction over at least 24 hours." We tried this method on three samples of adductor magnus muscle (in water acidified to pH 2.2 with nitric acid) and obtained values of from 23 to 28 milliequivalents

of chlorine per kilogram of muscle. Krogh's values varied from 11 to 23 milliequivalents, and he states that different muscles gave different results. The adductor magnus muscle has a thin connective-tissue sheath and would presumably give higher values by Krogh's method than would muscles whose thick sheaths would tend to prevent the outward diffusion of chloride. In one test, instead of merely heating the muscle and its surrounding water to boiling, we kept a piece of adductor magnus muscle in water on a steam water bath for an hour and then let the preparation stand over night. Following such treatment we obtained more chloride in the water extract (40 milliequivalents per kilogram of muscle). It is only logical to assume that, if the muscle is ground up and then extracted for hours with hot water, still higher chloride values will be obtained.

Our values for the chloride content of frog muscle, though much higher than the values accepted in the literature, are perhaps lower than the true values. It is not at all unlikely that, following our extractions, some chloride still remained in the muscle.

If our values for the chloride content of muscle are accepted, we can conclude even without further evidence that the fibers must contain chloride, for, if the extracellular space contains chloride in the same concentration as blood (Maurer, 1938) and if from our figures the whole muscle has a concentration of chloride over half that of blood, then over half of the muscle is chloride space.

On the basis of our figures for the chloride content of fibers and the whole muscle, it is possible by a simple algebraic calculation to compute the percentage of extracellular space. If the blood or the extracellular space contains 82 milliequivalents of chlorine per liter (Maurer, 1938), then, on the basis of the water-extract values, the extracellular space is 25 per cent. Or we may take a somewhat higher value for the chloride of blood and tissue spaces, such as we can obtain from Hill and Kupalov's (1930) value for the vapor pressure, if we subtract the value for bicarbonate (not bound to protein) as estimated by Barkan, Broemser, and Hahn (1921).[2] This would give a blood chloride content of 112 milliequivalents per liter, and the extracellular space would then be 14 per cent. For similar calculations based on the chloride content determined after acid digestion according to Van Slyke, we would obtain a value of 12 per cent if we used the Maurer value for blood and 7 per cent if we used the higher blood chloride value.

We do not regard these estimates of extracellular space as having any value. Admittedly, our chloride values are too low; and this is especially true of the values for the isolated fibers, for these fibers were dissected and washed in chloride-free Ringer's solution, and, during this period of dissection and washing, some chloride must have escaped. Such escape was probably more rapid through the cut ends of the fibers, but presumably there was also chloride loss across the intact membranes of the cells. If we assume an error of only 10 per cent, then our value for extracellular space as calculated from the chloride in water extracts and Maurer's values for the chloride in tissue spaces would be cut from 25 per cent to 16 per cent.

Our results indicate that some of the chloride in muscle cells is bound, but it is possible that the binding may be an artifact which occurs in the course of the nitric acid treatment. The presence of bound chloride in certain types of tissues was suggested by Hanke

[2] Barkan, Broemser, and Hahn determined the bicarbonate content of frog blood serum after precipitation of proteins by alcohol to be 0.15 per cent sodium bicarbonate. However, the alcohol did not precipitate all the proteins, and this was believed to introduce an error. Accordingly, the sodium bicarbonate concentration was estimated as being approximately 0.1 per cent. Ordinarily, much higher concentrations of bicarbonate are thought to be present in vertebrate blood (see, e.g., Van Slyke, Stillman, and Cullen, 1919).

and Donovan (1927). These authors give no values for muscle. Bound chloride has also been claimed for blood plasma; see, for example, Falta and Richter-Quittner (1918), who believed that fibrinogen combined with chloride. Peters and Man (1934) showed that lipids extracted from blood serum contained small amounts of chlorine, but it is not certain that in the normal blood the chlorine is actually bound to the lipids.

Rusznyák (1920) studied the chloride content of ultra-filtrates of human blood plasma and blood serum, and his analyses showed higher chloride values for the ultra-filtrates than for the unfiltrated blood. This might be interpreted as being due to the binding of chloride by protein in the blood, but Rusznyák himself thought that the lower concentration of chloride in the blood itself was due to the presence of colloidal material which acted as nonsolvent space. In his analyses Rusznyák used the von Korányi (1897) method. This is essentially similar to the Van Slyke method, except that potassium permanganate is added to the nitric acid in which ashing occurs. Rusznyák notes parenthetically that, with higher concentrations of permanganate, noticeably greater amounts of chloride are found in the analysis. This suggests that the original von Korányi method might prove superior to the Van Slyke modification of it. We have no information on this point.

We believe that the accepted views concerning the chloride content of tissues need revision. Muscle cells certainly contain appreciable amounts of chloride. Moreover, published values of the chloride content of muscle (and perhaps of other tissues) are too low.

SUMMARY

Isolated frog muscle fibers were dissected out in chloride-free solutions and were then analyzed for chloride content. Two types of methods were employed, and in both cases the fibers contained about 80 per cent as much chloride as the whole muscle. Water extracts of muscle fibers and of whole muscle gave higher chloride values than extracts obtained after wet-ashing with concentrated nitric acid. The latter procedure is the one usually employed in analyses of tissue chlorides, but it is apparently not suitable for muscle.

LITERATURE CITED

BARKAN, G.; BROEMSER, P.; and HAHN, A. 1921. Eine gepufferte Durchströmungsflüssigkeit für die überlebende Froschniere. Zeitschr. f. Biol., **74**:1.

BOURDILLON, J. 1937. Distribution in body fluids and excretion of ingested ammonium chloride, potassium chloride, and sodium chloride. Amer. Jour. Physiol., **120**:411.

BOYLE, P. J., and CONWAY, E. J. 1941. Potassium accumulation in muscle and associated changes. Jour. Physiol., **100**:1.

BOYLE, P. J.; CONWAY, E. J.; KANE, F.; and O'REILLY, H. L. 1941. Volume of interfibre spaces in frog muscle and the calculation of concentrations in the fibre water. Jour. Physiol., **99**:401.

CONWAY, E. J.; KANE, F.; BOYLE, P.; and O'REILLY, H. 1939. Localization of electrolytes in muscle. Nature, **144**:752.

DEAN, R. B. 1941. The determination of chloride in single isolated muscle fibers. Jour. Biol. Chem., **137**: 113.

EICHELBERGER, L., and MCLEAN, F. C. 1942. The distribution of calcium and magnesium between the cells and the extracellular fluids and skeletal muscle in dogs. Jour. Biol. Chem., **142**:467.

FALTA, W., and RICHTER-QUITTNER, M. 1918. Studien über die Faserstoffgerinnung. I. Über das Vorkommen von gebundenem Chlor im Blutplasma. Biochem. Zeitschr., **91**:381.

FENN, W. O. 1936. Electrolytes in muscle. Physiol. Rev., **16**:450.

FISHER, R. B., and SUBRAHMANYAN, V. 1939. The diffusion of chloride and glucose into frog muscle. Jour. Physiol., **97**:233.

GERSH, I. 1938. Improved histochemical methods for chloride, phosphate-carbonate and potassium applied to skeletal muscle. Anat. Rec., **70**:311.

HANKE, M. E., and DONOVAN, P. B. 1927. Organic chlorides of tissues and possible relation to gastric hydrochloric acid formation. Proc. Soc. Exper. Biol. and Med., **24**:580.

HARRISON, H. E.; DARROW, D. C.; and YANNET, H. 1936. The total electrolyte content of animals and its probable relation to the distribution of body water. Jour. Biol. Chem., **113**:515.

HILL, A. V., and KUPALOV, P. S. 1930. The vapour pressure of muscle. Proc. Roy. Soc., London, B, **106**:445.

KORÁNYI, A. VON. 1897. Physiologische und klinische Untersuchungen über den osmotischen Druck thierischen Flüssigkeiten. Zeitschr. f. klin. Med., **33**:1.

KROGH, A. 1937. Osmotic regulation in the frog (*R. esculenta*) by active absorption of chloride ions. Skandinav. Arch. f. Physiol., **76**:60.

LISON, L. 1936. Histochimie animale. Paris.

MACALLUM, A. B. 1905. On the nature of the silver reaction in animal and vegetable tissue. Proc. Roy. Soc., London, B, **76**:217.

MANERY, J. F., and BALE, W. F. 1941. The penetration of radioactive sodium and phosphorus into the extra- and intracellular phases of tissues. Amer. Jour. Physiol., **132**:215.

MAURER, F. W. 1938. Isolation and analysis of extracellular muscle fluid from the frog. Amer. Jour. Physiol., **124**:546.

OVERTON, E. 1902. Beiträge zur allgemeinen Muskel- and Nervenphysiologie. II. Ueber die Unentbehrlichkeit von Natrium- (oder Lithium-) Ionen für den Contractionsact des Muskels. Arch. f. d. ges. Physiol., **92**:346.

PETERS, J. P. 1935. Body water. Springfield, Ill.

PETERS, J. P., and MAN, E. B. 1934. Lipoid-chlorine in serum. Jour. Biol. Chem., **107**:23.

RUSZNYÁK, S. 1920. Physikalisch-chemische Untersuchungen an Körperflüssigkeiten: Der Zustand des Chlors in Serum und Plasma. I. Biochem. Zeitschr., **110**:60.

SCOTT, G. H., and PACKER, D. M. 1939. An electron miscroscope study of magnesium and calcium in striated muscle. Anat. Rec., **74**:31.

TRUAX, F. L. 1939. The equality of the chloride space and the extracellular space of rat liver. Amer. Jour. Physiol., **126**:402.

VAN SLYKE, D. D. 1923. The determination of chlorides in blood and tissues. Jour. Biol. Chem., **58**:523.

VAN SLYKE, D. D.; STILLMAN, E.; and CULLEN, G. E. 1919. Studies in acidosis. XIII. A method for titrating the bicarbonate content of the plasma. Jour. Biol. Chem., **38**:167.

WIGGLESWORTH, V. B. 1937. A simple method of volumetric analysis for small quantities of fluid: estimation of Cl in 0.3 μl of tissue fluid. Biochem. Jour., **31**:1719.

WILSON, D. W., and BALL, E. G. 1928. A study of the estimation of chloride in blood and serum. Jour. Biol. Chem., **79**:221.

EFFECT OF ANOXIA ON TRIGEMINAL REFLEXES IN CAT FETUSES[1]

W. F. WINDLE, R. F. BECKER, RUTH RHINES, AND ELIZABETH J. COWGILL
Anatomical Laboratories, Northwestern University Medical School

MAMMALIAN fetuses can be made to respond to changes in an artificial external environment before the middle of the gestation period. Reflex movements of the forelimbs and head are elicitable in cat embryos at experimental Cesarean section on the twenty-fourth day after insemination (Windle, Orr, and Minear, 1934). The first clear evidence that fetuses respond to induced alteration of their internal environment is the respiratory movement occurring when anoxial conditions are produced experimentally at about 32 days of gestation (Windle, Monnier, and Steele, 1938). Previous to this, internal environmental changes can alter responsiveness to external stimuli (Windle and Becker, 1940*a*).

Various investigators have proposed that oxygen want, or asphyxia with carbon dioxide accumulation, may stimulate groups of neurons, release them from higher inhibitory influences, or simply depress their activity altogether. Angulo y Gonzalez (1930) believed that asphyxia caused the fetus to recapitulate inversely its behavioral ontogeny by stimulating growing neurons. However, it may be doubted that the responses he observed were reflexes, because they have been reproduced in dying embryos placed in solutions of ionic imbalance (Windle, 1939). Barcroft and Barron (Barron, 1941) rejected the concept that it is metabolic carbon dioxide which acts as a stimulating agent. Experiments in sheep fetuses led them to suggest that anoxemia depressed newly developed inhibitory centers of the brain, rendered them nonfunctional, and left older motor neuron groups to function in a manner characteristic of younger fetuses (Barcroft and Barron, 1939). Windle and Becker (1940*a*) found that anoxemia first enhanced, then suppressed, individual fetal reflexes. Appendicular movements—as early as any reflexes to appear under reasonably good physiologic conditions—were abolished before more massive trunk and neck reactions. There was little correlation between age of a pattern of movement and length of time the response persisted after clamping the umbilical cord. Anoxia appeared to convert unit reflexes into mass reactions. Porter, Blair, and Bohmfalk (1938) found that slight anoxia and even asphyxia increased the number of adult motor units responding to a given stimulus, and transient stimulation of the nervous system by progress anoxia has been demonstrated by Sugar and Gerard (1938).

From time to time it has been observed that embryos or small fetuses respond in one way during the early part of an experiment and in another way later in the same experiment as the reaction changes as anoxia develops. This led to a further study of reflex reversals and their relation to anoxemia.

MATERIAL AND METHODS

Observations were made on 125 embryos and fetuses of 36 cats of between 24 and 63 days' gestation. The pregnant animals were decerebrated by ligating internal carotid and

[1] Aided in part by grants from The John and Mary R. Markle Foundation and the Women's Faculty Club of Northwestern University Medical School.

basilar arteries under ether anesthesia. This was done about an hour before observations were to be started, in order that anesthetics and narcotics could be dispensed with during the experiments.

The decerebrated cat was immersed in a bath containing Locke's solution at body temperature and the uterus delivered through an abdominal incision. One at a time the fetuses were exposed quickly and stimulated. Circulation was maintained to the intact placenta during the first 1–10 minutes of each experiment. Later the umbilical cord was clamped or the placenta was separated from the uterus to study effects of anoxia.

Various types of stimuli were used. Some were light, e.g., a single hair, a probe, or a small camel's hair brush; others were strong, e.g., a needle point or faradic shock from an inductorium with 1.5 volts on the primary coil. The effectiveness of a stimulus seemed to depend less upon its strength than upon the physiologic condition of the specimen and size of the area of surface stimulated. Sometimes stimulation of a single point failed to cause a response, but touching a large area in the same region with a blunt probe or brush led to a reaction. Using insulated nichrome wire electrodes, the bare tips of which were spaced about 1 mm. apart, faradic shocks from the secondary coil set at 6 or 8 cm. were usually effective before the physiologic condition of the fetus began to decline markedly. Thereafter, a setting of 3 or 4 cm. was required. That stronger shocks induced a reflex when weaker ones failed probably depended on spreading of current to a greater number of nerve endings.

Special attention was given to primary responses resulting from stimulating regions on the head, specifically the tip of the nose, side of the nose or face, upper eyelid region or cornea, ear, and lower jaw or lip. Complex reactions were obtained from the older fetuses. Nevertheless, all began with a movement of the head to one side or the other, even though other head, trunk, and limb movements followed in quick succession. Principal attention will be directed to the component which initiated the complex series of activities, and this will be referred to as the "initial response."

RESULTS

It is imperative to understand the limitations of the experimental method before considering results. It is tempting to assume that, so long as an embryo is attached by its placenta to the uterus, is kept warm, and is in a saline bath, its physiological condition is "normal" and the behavior which can be elicited from it represents that which occurs *in utero*. Nothing could be further from the truth. The assumption has come about because of prevalent misconceptions that anoxemia is normal in the fetus, that the blood from the placenta is highly unsaturated to begin with, and that it is mixed in the fetal heart with venous blood before being directed to the fetal brain in an even more unsaturated state. What, then, if incision of the uterus does slightly disturb physiologic relations? Throughout the part of the gestation period with which the present experiments are concerned the fetal blood actually becomes nearly saturated with oxygen in the placenta (Barcroft, Kennedy, and Mason, 1940). Because of certain shunts in the heart, this blood reaches the fetal heart muscle and brain without much loss of oxygen (Windle and Becker, 1940*b*). It is difficult to obtain blood samples from the fetus without interfering with the placental exchange, although it has been done. It has been impossible to obtain fully oxygenated samples after manipulating and opening the uterus to deliver the fetus. Therefore, observations on fetal behavior after delivery are bound to be affected by some degree of

anoxemia. If one would learn how the fetus behaves within the uterus, one must be prepared to cut and make observations quickly.

By so doing it was found that the cat fetus was distinctly unresponsive to mechanical and electrical stimuli throughout the period from 26 days to full term. The umbilical veins were brilliantly red, but within a few seconds the color began to darken. As it did so, the fetus started to respond to stimulation, and soon "spontaneous" movements appeared. The initial lack of responsiveness was observed time and again and was so striking that it sometimes led the observers to look twice to make certain the fetal heart was beating. One of the present investigators observed a similar phenomenon in the human embryo. At the operating table when the umbilical-vein blood was red, specimens at first failed to respond to stimulation, but after the placenta had been delivered they became irritable. In experiments with cats not included in the present series attempts were made to stimulate the fetuses with needles passed through the intact abdomen. Rarely were responses obtained, and when present they were slight and of brief duration (Windle, 1940); anoxia enhanced irritability. One should not imply that the fetus makes no movements before it is born. It is well known that it does so, especially toward the latter part of fetal life. But it is certainly less irritable before it is delivered than soon thereafter.

Reflexes with which the present report deal were obtained after fetuses had been exposed and after at least a partial anoxemia prevailed. To distinguish these from the reactions occurring after the placental circulation had been interrupted we shall speak of them as "preanoxic."

Results of stimulating various regions on the face are summarized in Table 1. The earliest reflex movement of the head resulted from stimulation of zones supplied by maxillary branches of the trigeminal nerves in cat embryos 23.5–24 days after insemination. At first it was possible to obtain a response more readily by stimulating the tip of the nose, i.e., both maxillary nerves, then either side alone. The reflex was a quick backward movement of the head. Throughout the series of experiments under consideration this reaction was obtained before the cord was clamped but during partial anoxemia. Occlusion of the umbilical vessels or separation of the placenta from the uterus led to a change in this reflex at about 32 days. When anoxia had been prolonged for 3–10 minutes or more, a stimulus applied to the nose resulted in a forward flexion of the head. This reversal was similar to that observed in chicks and sheep, as well as in cat fetuses during depression of respiratory efforts by marked anoxemia.

Stimulation of the region supplied by only one maxillary nerve before clamping the cord elicited a contralateral movement of the head from about 25–27 days. From 27 to 29 days the initial response was a movement to the homolateral side. From 30 days until 63 days it was again contralateral. Thus two changes took place in the preanoxic reaction. After the umbilical cord had been clamped, it was possible to obtain a contralateral reflex at 24 days, and an initial homolateral head reflex manifested itself at 26 days. This began to change to contralateral flexion again at 29 days; but a homolateral response was sometimes encountered during anoxia as late as 36 days, i.e., 6 days after the preanoxic initial reaction had become fixed as an avoidance response.

Reflexes followed stimulation of the upper eyelid and corneal regions before they could be obtained from the forehead. At 25–26 days after insemination the initial preanoxic reaction was a homolateral movement of the head. A secondary contralateral flexion

sometimes occurred after this initial movement as early as 27 days. At 36 days the initial movement became contralateral and remained so throughout older stages. During anoxia a homolateral response could be obtained easily at 25 days. This was the only reflex until a movement of the head away from the stimulus began to appear at 27 days. The latter became well established by 31 days.

It will be seen that, in case of the maxillary and ophthalmic reactions, changes came about after the fetuses had been rendered anoxic and these changes were like those which

TABLE 1*

INITIAL RESPONSES OF CAT FETUSES

Approximate Average			Regions Stimulated									
Age (Days)	Length (Mm.)	Weight (Gm.)	Tip of Nose		Side of Face		Above Eye		Ear		Lower Lip	
			P	A	P	A	P	A	P	A	P	A
24	15	0.2	E	E		C						
25	16	0.4			C			H				
26	18	0.5			↓	↓ H	H			C	C	C
27	20	0.7			H		↓		C	↓	↓	↓
28	22	0.9			↓	C			↓ H		H	
29	25	1.1			C							
30	28	1.4						↓				
31	33	2.1		↓								
32	40	2.8		F								
34	44	3.2										
35	49	5.3										
36	55	6.5				↓	↓ C		↓ C			
40	62	10.0										
56	110	41.0										
63	123	79.0	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓

* P indicates the preanoxic response occurring before clamping the umbilical cord; A indicates the response after anoxia was induced by clamping the cord. The letters E, F, C, and H refer to extension, flexion, contralateral, and homolateral. The solid portions of the arrows indicate periods during which responses were relatively fixed; the broken portions of the arrows denote variability or infrequency of the type of response in question.

occurred before clamping the cord but they took place a little earlier in the gestation period. The maxillary response began to change a second time during severe anoxia before it changed under preanoxic conditions. Certain similarities between these reversals and the "Porter Phenomenon" will be noticed (Rosenblueth and Ortiz, 1936; Rosenblueth, Klopp, and Simeone, 1938).

A different series of observations followed stimulation of the regions which receive innervation from branches of the mandibular nerves. Only on the lower lip could we feel reasonably certain that light stimuli were confined to this nerve; elsewhere the chances for spreading were great. Nevertheless, early reactions from the ear were quite similar to those from the lip. From both of these regions the earliest initial preanoxic responses

were contralateral head flexions at 27 days. From the ear at 29 days and from the lower lip at 30 days homolateral reflexes were obtained. By 36 days the ear response again changed to contralateral, but stimulation of the lower lip induced a homolateral movement at all later stages. This homolateral movement was apparently part of the feeding reaction pattern.

During the anoxia following clamping of the umbilical cords, responses from the ear and lower lip appeared earlier (at 26 days). No true reversals were observed with certainty during anoxia. It is believed that apparent reversals (see Table 1) were due entirely to spreading of stimuli to adjacent regions (maxillary and spinal nerves). From the posterior part of the jaw the reaction obtained at all ages and under all conditions was movement of the head away from the stimulus. Occasionally a homolateral reaction was encountered at the time that the preanoxic maxillary response was homolateral, but spreading of the stimulus could have occurred here. The type of reflex obtained from the angle of the jaw was sufficiently different from that from the lower lip (definitely mandibular) and the external ear (probably mandibular) to suggest that the third division of the trigeminal was not the afferent nerve concerned in it.

DISCUSSION

Results may be interpreted as follows: On the twenty-fourth day after insemination the nervous system of the cat fetus reaches a stage in development in which a limited amount of function is possible. In pyridine silver–stained cat embryos 14 mm.–15 mm. long a few central trigeminal connections could be observed. Homolateral interneurons appeared to be no more numerous than commissural elements in earlier stages in development (Rhines and Windle, 1941). From the twenty-fourth day onward the repertoire of behavioral capabilities increased. In the period between 25 and 30 days interneurons became more numerous in the reticular formation. It is not surprising that marked functional changes accompanied and followed this expansion in the central nervous system.

Although the embryo was capable of responding to stimuli after it had been exposed during experiments, it was singularly quiescent *in utero*. As long as normal physiologic conditions, including a high degree of oxygenation of the fetal blood reaching the brain, prevailed, reflexes could be induced only by employing strong stimulation of numerous afferent neurons. It would seem that the nervous system is endowed with high thresholds under these conditions. Responses which were elicited tended to be of the unit type rather than mass movements. Many afferent neurons must have been conducting before one motor unit would respond.

During the partial anoxemia which developed in consequence of opening the uterus to observe the fetus (and even in the best of experimental conditions some degree of anoxemia is unavoidable) neural thresholds declined. A stimulus which previously elicited no response then called forth a reflex. Anoxia exerts a stimulating effect on the nervous system at first (Porter, Blair, and Bohmfalk, 1938; Sugar and Gerard, 1938); in the fetus this effect appeared to last longer than it does in the adult. During mild anoxemia the reflexes tended to be of the unit type, but their nature began to change when experiments were prolonged.

Severe anoxia induced by clamping the umbilical cord led to qualitative and quantitative changes in response to stimulation. It caused unit responses to become mass movements, often convulsive or rhythmic in character (Windle and Becker, 1940*a*). Ultimately, anoxia brought on depression and death. However, in fetuses and young animals

survival times are longer than in the adult (Kabat and Dennis, 1939; Fazekas, Alexander, and Himwich, 1941).

Rapid growth of nervous elements may account in part for progressive reflex reversals which were observed in the cat fetuses. The number of neurons, especially interneurons, increases, processes grow in length, and their connections (collateral fibers) multiply many times. Changes in the preanoxic response to stimulating a particular group of afferent neurons may be explained on the basis of shifting predominance of associational and commissural interneuron connections. The differences among responses from stimulating the several branches of the trigeminal nerve may have been due to variations in level of termination in the brain stem and to spatial relations in the spinal tract. For example, the ophthalmic fibers pass farther down the medulla oblongata than those of other branches. They overlap spinal afferent neurons in the dorsal funiculus, coming to lie at the ventrolateral border of the latter. There they are in better position to effect connections with associational than with commissural interneurons (Windle, 1934). Consequently, the first responses were homolateral. As collaterals of the ophthalmic fibers grow out at higher levels, an increasing number of connections are made with commissural elements in the medulla oblongata. Ultimately, these take numerical dominance over the lower and earlier direct connections, and the preanoxic response to afferent stimulation changes to contralateral (36 days).

But why did some of the trigeminal reflexes become reversed at an earlier time under influences of severe anoxia? Perhaps the answer to this question is the same as that to the question: Why did anoxemia make it possible to obtain responses with a stimulus which was too weak to induce a reflex under normal conditions? Young synapses would seem to have thresholds which are high but which become lowered more readily by anoxia than those of older synapses. In the case of the ophthalmic responses many contralateral connections had been made by the thirty-first day and even earlier. But only under duress of anoxia did they take dominance over the older connections of lower threshold.

Prolonged anoxia certainly impaired reflex activity and ultimately abolished all nervous functions. During this process it is assumed that chance played some role in determining which neurons were affected first. It was not always the older reaction which was retained while the newer patterns were broken up. Forelimb reflexes—at least as early as any to appear in prenatal life—were abolished by anoxia before head and trunk reflexes (Windle and Becker, 1940*a*). Respiratory movements developed relatively late but were the most resistant of all patterns. Anoxia brought about such directional changes as flexion instead of extension and homolateral head movement instead of an avoidance response; yet the movement during anoxia was not one which had appeared earlier in gestation under more nearly normal circumstances. The theory of Barcroft and Barron (1939) does not explain these phenomena adequately.

From results of stimulating the mandibular nerve it may appear that a reversion to a more primitive type of response did take place during severe anoxia. There is some doubt that this is the correct explanation, however. As in the case of the afferents from the limbs, the connections made by mandibular neurons were less resistant to anoxia than those of the other trigeminal branches. The mandibular responses always disappeared before others, maxillary reflexes being the last to go. When stimulation of the mandibular region was strong enough to elicit any response during anoxia, it probably did so because

it spread to adjacent spinal nerve endings or to the maxillary regions; both the spinal and the maxillary nerves gave contralateral responses during anoxia during the period in question.

SUMMARY

Reflexes initiated by stimulating the nose, side of the face, eye, lower lip, and ear of cat fetuses up to 63 days' gestation were studied with particular emphasis upon the initial components of head movement. The pregnant cats were decerebrated and experiments subsequently conducted without anesthetics or narcotics. Circulation of the fetal blood to the intact placenta was maintained while responses to stimulation were being observed. Later the umbilical cord was clamped to induce anoxia in the fetus.

Fetuses were relatively unresponsive when first delivered; but partial anoxemia, attendant upon opening the uterus, led to increased irritability in a few seconds or minutes. In early stages reflexes appeared under duress of prolonged anoxia, although they had been absent during partial anoxemia. Reflexes were elicited at 23.5–25 days of gestation more readily by stimulating a large surface area and activating many receptors than by stimulating a point and activating a few receptors.

During the course of development, reflexes resulting from stimulation of any given group of receptors in the head before clamping the umbilical cord changed once or twice. It was suggested that these reversals came about in consequence of a fluctuating preponderance of commissural or associational interneuron connections in the developing brain.

Similar reversals occurred at earlier stages in development under anoxia when maxillary and ophthalmic regions were stimulated. Thus were anticipated, as it were, the changes about to take place under better physiologic conditions. Anoxia enhanced the irritability of neurons at first and ultimately abolished all reflex activities. It exerted some selective action in doing so, but the first reflexes to appear ontogenetically were not necessarily the last to disappear during anoxia.

REFERENCES CITED

ANGUELO Y GONZALEZ, A. W. 1930. Endogenous stimulation of albino rat fetuses. Proc. Soc. Exper. Biol. and Med., **27**:579 (see also Anat. Rec., Suppl., **58**:45, 1934).

BARCROFT, J., and BARRON, D. H. 1937. Movements in midfoetal life in the sheep embryo. *Jour. Physiol.*, **91**:329.

———. 1939. Movement in the mammalian foetus. Ergebn. d. Physiol., **42**:107.

BARCROFT, J., KENNEDY, J. A., and MASON, M. F. 1940. Oxygen in the blood of the umbilical vessels of the sheep. Jour. Physiol., **97**:347.

BARRON, D. H. 1941. The functional development of some mammalian neuromuscular mechanisms. Biol. Rev., **16**:1.

FAZEKAS, J. F., ALEXANDER, F. A. D., and HIMWICH, H. E. 1941. Tolerance of the newborn to anoxia. Amer. Jour. Physiol., **134**:281.

KABAT, H., and DENNIS, C. 1939. Resistance of young dogs to acute arrest of the cephalic circulation. Proc. Soc. Exper. Biol. and Med., **42**:534.

PORTER, E. L.; BLAIR, R. K.; and BOHMFALK, S. W. 1938. Facilitation of reflex action in the spinal cord following asphyxia of the cord from cyanide and increased intraspinal pressure. Jour. Neurophysiol., **1**:166.

RHINES, RUTH, and WINDLE, W. F. 1941. The early development of the fasciculus longitudinalis medialis and associated secondary neurons in the rat, cat, and man. Jour. Comp. Neurol., **75**:165.

ROSENBLUETH, A., and ORTIZ, T. 1936. The crossed respiratory impulses to the phrenic. Amer. Jour. Physiol., **117**:495.

ROSENBLUETH, A., KLOPP, C. T., and SIMEONE, F. A. 1938. A further study of the crossed phrenic phenomenon. Jour. Neurophysiol., **1**:508.

SUGAR, O., and GERARD, R. W. 1938. Anoxia and brain potentials. Jour. Neurophysiol., **1**:558.

WINDLE, W. F. 1934. Correlation between the development of local reflexes and reflex arcs in the spinal cord of cat embryos. Jour. Comp. Neurol., **59**:487.

———. 1939. Calcium and potassium deficiency as possible causes of certain delayed fetal movements. Physiol. Zoöl., **12**:39.

———. 1940. Physiology of the fetus. Philadelphia: W. B. Saunders.

WINDLE, W. F., and BECKER, R. F. 1940*a*. Relation of anoxemia to early activity in the fetal nervous system. Arch. Neurol. and Psychiat., **43**:90.

———. 1940*b*. The course of blood through the fetal heart. Anat. Rec., **77**:417.

WINDLE, W. F., MONNIER, M., and STEELE, A. G. 1938. Fetal respiratory movements in the cat. Physiol. Zoöl., **9**:425.

WINDLE, W. F., ORR, D. W., and MINEAR, W. L. 1934. The origin and development of reflexes in the cat during the third fetal week. Physiol. Zoöl., **7**:600.